Milos Brankovic

HEART KIDNEY INTERACTIONS

and their temporal relationships


Before you sleep,
read something
that is exquisite
and worth remembering.
Desiderius Erasmus

Milos Brankovic

HEART KIDNEY INTERACTIONS

and their temporal relationships

Colophon

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ABOUT THE PARTS' IMAGES

Four Parts' images – "Fluidal connection", "Symbiosis", "Whirlpool", and "Still life" – illustrate the notions that came into my mind during the preparation of this thesis. I could not describe these ideas through words, but as mental figures of a kind that can only be illustrated by presenting them as visual images.

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HEART-KIDNEY INTERACTIONS and their temporal relationships

Hart-nier interacties
en hun temporele relaties

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NOTICE

The knowledge and the body of evidence within the field of medicine grow constantly. As new investigations and experience broaden our understanding of the human (mal)functioning, changes in the research methodology, concepts, and clinical practice may follow. Therefore, healthcare providers and researchers should always rely on their own knowledge and experience in evaluating and using any information, method, or experiment discussed in this thesis.

MANUSCRIPTS BASED ON THE STUDIES DESCRIBED IN THIS THESIS

Chapter 2

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Chapter 3

Brankovic M, Kardys I, Steyerberg EW, Lemeshow S, Markovic M, Rizopoulos D and Boersma E. On the Understanding of Statistical Interaction for Clinical Investigators. (submitted)

Chapter 4

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Chapter 5

Brankovic M, Akkerhuis KM, Hoorn EJ, van Boven N, van den Berge JC, Constantinescu A, Brugts JJ, van Ramshorst J, Germans T, Hillege H, Boersma E, Umans V and Kardys I. Glomerular Decline and Progressive Tubular Damage in Chronic Heart Failure: Clinical Determinants and Combined Value for Prognosis The Bio-SHiFT Study. (submitted)

Chapter 6

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Chapter 7

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Chapter 9

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Chapter 10

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Chapter 11

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Chapter 12

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Chapter 13

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CHAPTER 1

INTRODUCTION

"Disease is very old and nothing about it has changed. It is we who change as we learn to recognize what was formerly imperceptible."
Jean-Martin Charcot

The heart–kidney interactions described in this thesis are based on our experience derived from clinical studies conducted among patients with heart failure (HF) and those with ischemic heart disease (IHD). The thesis describes heart–kidney interactions not only as the organs' interplay assessed at a single moment in time as is commonly done, but also their temporal relationships over time preceding adverse clinical events. This is important to note because these temporal patterns have so far received insufficient attention, mainly due to the methodological limitations of previous studies. However, these patterns are inherently linked to the progression of the conditions that affect both the heart and the kidneys such as HF and atherosclerosis.

HF and IHD are global health problems that pose a great burden on patients, healthcare systems, and society in general.[1,2] Besides their high prevalence, HF and IHD are the leading causes of death worldwide, with HF being also the leading cause of re-hospitalization.[1-4] One of the common denominators in both conditions is kidney dysfunction, where approximately half of patients with HF and one fourth of those with IHD suffer from chronic kidney disease (CKD).[5,6] Importantly, the kidney disease not only co-exists, but also interacts with cardiac diseases thereby further reducing patients' survival.[1,5] Interestingly, CKD patients are six times more likely to die of cardiovascular diseases than to reach end-stage renal disease.[7] Taken together, it is clear that heart–kidney interactions are bidirectional and that their identification, assessment and proper management still remain challenging.

"A scientist does not (only) aim at the immediate results. He does not expect that his advanced ideas will be readily taken up. His work is like that of a planter – for the future. His duty is to lay the foundation for those who are to come, and point the way."

Nikola Tesla

This book is divided into four main parts: "Methodological concepts", "The role of the kidneys in heart failure and beyond", "Implications of renal function for ischemic heart disease", and "Lessons learned from clinical practice". Each part contains chapters that explain specific aspects of heart–kidney interactions, but also build on the preceding chapter. In chapter 2, the concepts of the "temporal patterns" and the "personalized risk assessment" are described, which have not been extensively explored in medicine. These concepts were subsequently applied in clinical studies reported in chapters 4 to 9. Briefly, in these chapters we examined individual temporal trajectories of multiple blood and urine markers to derive estimates of patient-specific (i.e., personalized) prognosis. For this purpose, we assessed the marker's levels, but also the slope (i.e., rate of change) of the marker's trajectory, and the cumulative effect of all values that the marker has taken until the time of the assessment. These aspects are valuable as they provide us with a comprehensive picture of disease dynamics and the patient's prognosis.

"I did not care to get a diploma, but to get qualified as an independent scientist. That was my goal! I have realized that the true science makes only what is of general scientific significance."

Milutin Milankovic

This thesis was guided by four main objectives. The first objective was to perform a critical appraisal of dynamic prediction modeling (chapter 2) and interaction testing (chapter 3) in clinical studies.

The second objective was to investigate how trajectories of glomerular and tubular renal compartments relate to each other over time preceding adverse clinical events, and how their individual and joint assessments relate to the prognosis of patients with chronic HF (chapters 4 and 5). Thereafter, we determined the predictive utility of temporal patterns of new HF biomarkers that are expected to emerge in the near future (chapters 6 to 8).

The third objective was to determine the implications of renal function for IHD. Specific aims included assessment of the evolution of renal function from its ini-

tial change during acute coronary syndrome (ACS) until stabilization, and investigating the predictive value of serial renal assessments in these patients (chapter 9). Moreover, we examined the relation of a potent glomerular marker, cystatin C– and a tubular marker, NGAL– with coronary atherosclerosis assessed in-vivo by intravascular ultrasound (IVUS) virtual histology and with patients' adverse outcomes (chapter 10).

The fourth objective was to evaluate different perspectives of clinical practice in HF patients with special attention to the kidneys. Specific aims included evaluation of the temporal relationships between guideline-recommended HF medication adjustments and multiple cardio-renal biomarkers, patients' functional status, and clinical outcomes in patients with chronic HF (chapter 11). In patients with end-stage HF, we investigated the relation of right heart and pulmonary hemodynamic parameters measured before heart transplantation with severity of postoperative acute kidney injury (chapter 12). Finally, we assessed the relation of renal dysfunction and anemia with short- and long-term survival in patients with acute HF using our registry data from 1985 to 2008 (chapter 13).

To meet the objectives, this thesis has combined several disciplines including methodologies of dynamic prediction modeling and interaction testing, utilization of modern assays based on –omics technologies for assessment of new biomarkers, sophisticated cardiovascular imaging techniques, and unique repeated-measures study designs. In the longer term, the results carry potential to contribute to reducing mortality- and hospitalization-rates in patients with acquired heart disease, improving their quality of life, and reducing healthcare costs.

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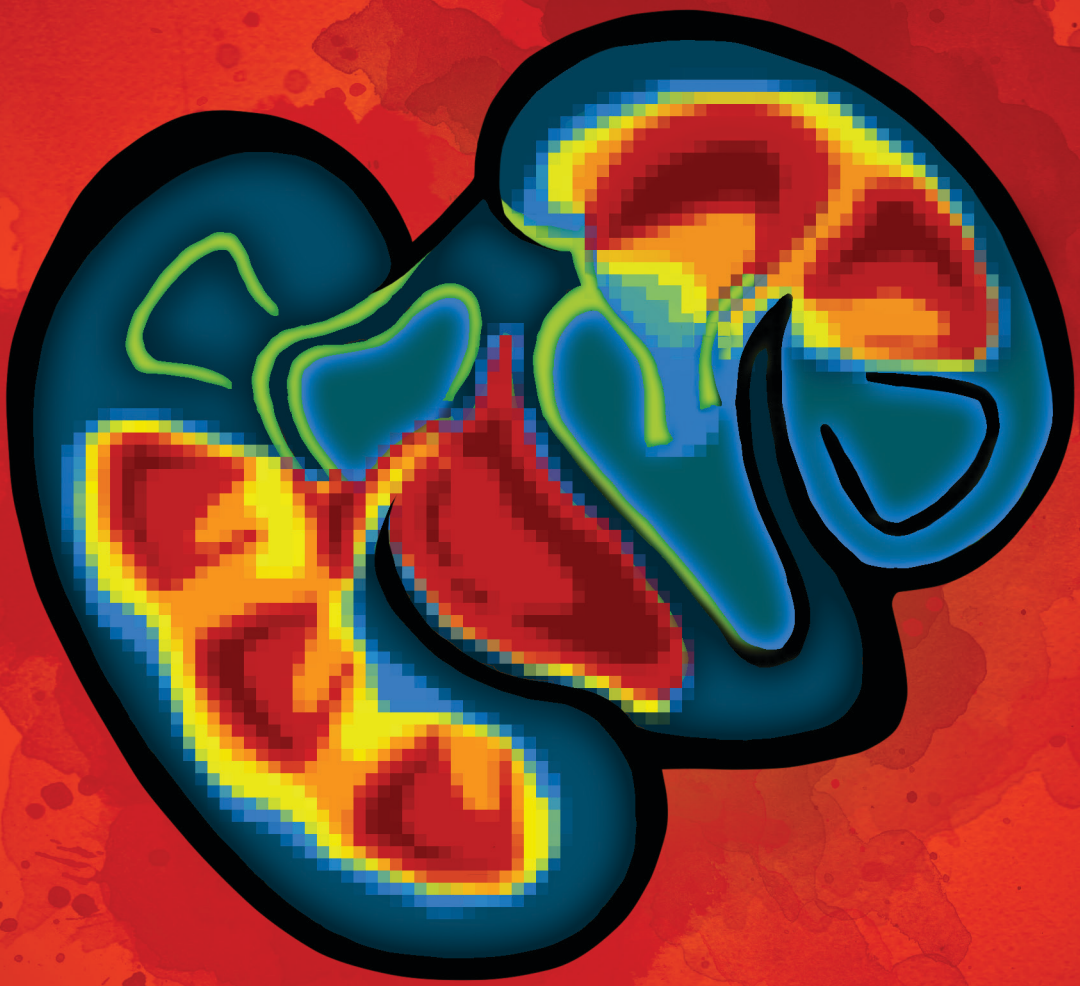


FLUIDAL CONNECTION

Part I

METHODOLOGICAL CONCEPTS

CHAPTER 2

Personalized Dynamic Risk Assessment – the Next Step in Prognostic Research

Milos Brankovic, Isabella Kardys, Ewout J. Hoorn, Sara Baart, Eric Boersma, Dimitris Rizopoulos

Kidney International, 2018; 94:214-217.

ABSTRACT

In medicine, repeated measures are frequently available (glomerular filtration rate or proteinuria) and linked to adverse outcomes. However, several features of these longitudinal data should be considered before making such inferences. These considerations are discussed and we describe how joint modeling of repeatedly measured and time-to-event data may help to assess disease dynamics and to derive personalized prognosis. Joint modeling combines linear mixed-effects models and Cox regression model to relate patient-specific trajectory to their prognosis. We describe several aspects of the relationship between time-varying markers and the endpoint of interest that are assessed with real examples to illustrate the aforementioned aspects of the longitudinal data provided. Thus, joint models are valuable statistical tools for study purposes, but also may help healthcare providers in making well-informed dynamic medical decisions.

INTRODUCTION

Application of longitudinal study designs to assess dynamics of medical conditions is currently gaining interest in general medical community and particularly in the fields of cardiology and nephrology.[1-5] Such study designs entail repeated measurements of biological markers (e.g., proteins in the blood or urine) over the time-course of the disease to infer patient prognosis.

As an illustrative example we will consider a study by Brankovic et al. who investigated how longitudinal trajectories of several glomerular and tubular markers in patients with chronic heart failure (HF) relate to their prognosis.[6] Samples were measured at fixed 3-month intervals during 2-year follow-up. Compared to studies that measured these markers at baseline only and related them to patient prognosis, the repeated-measures design utilized by Brankovic et al. carries several advantages.[7] Most importantly, it reflects disease dynamics better than the single-baseline assessment. However, when analyzing repeatedly measured biomarkers, the question arises how to properly relate them to prognosis.[7] To do this, several approaches can be utilized including time-dependent Cox model (TDCM).[8] Alternatively, joint models (JMs) of repeatedly measured and time-to-event data can be performed.

Reasons for choosing JMs over TDCM for estimating prognosis using time-varying markers are discussed below including data-collection, data-analysis, as well as the methodological concept behind JMs.

Data-collection

First, if repeated measurements are not collected at equally spaced time-points or not all patients have the same number of measurements, the longitudinal data are unbalanced.[9] This is often seen when treating physicians determine how often study-visits should take place for data to be taken. For example, Breidthardt[4] et al. studied whether worsening renal function (WRF) predicts mortality in patients admitted for acute HF. They defined WRF as in-hospital increase in serum creatinine ≥0.3mg/dl, and treating physicians determined the timing of serum creatinine sampling. Here, the sicker patients were likely to be monitored more closely (i.e., have more measurements taken) than the less sick patients. Consequently, the likelihood of finding WRF would increase in sicker patients. This unbalanced data-collection would falsely strengthen the association between WRF and mortality if this relation is modeled improperly.

Second, even when patient-visits occur at fixed time-points by a pre-specified study protocol, longitudinal data may become unbalanced. This occurs in three situations: when patients' measurements are not performed in the beginning but start later during follow-up ("late entry"), when patients skip some of the scheduled visits ("intermittent missing"), or when patients withdraw before the study ends ("early dropout").[7] In all situations, the longitudinal data become unbalanced because of missing values. Importantly, if the reason for the missing values is related to patients' survival (e.g., patient misses visits because of deteriorating condition), TDCM becomes inadequate because it assumes that missing values are independent of survival.[7] For example, Li et al. studied longitudinal creatinine-based glomerular filtration rate (GFR_{Cr}) trajectory in the African American Study of Kidney Disease in Hypertension (AASK) trial.[10] Here, 23% of patients were excluded because they withdrew before collecting a sufficient number of measurements. In the majority, the reasons for withdrawal were related to their time-to-event as they died or were started on renal replacement therapy (RRT) before obtaining sufficient serum creatinine measurements.

Data-analysis

Covariates measured (or collected) on patients are internal (i.e., endogenous) predictors. This is important to note because for any internal predictor (i.e., biomarker) future measurements potentially depend on the patient's survival which should be considered when analyzing such covariates.[11,12] This is due to two reasons: patients have to be alive and present at study-visits for markers to be measured, and markers' values might be affected by his/her condition up to that visit.[7] Additionally, internal predictors are biologically subjected to variability and can be measured with error.[7] Examples of such predictors are serum creatinine, body mass index, echocardiography measurements, or proteinuria.

TDCM cannot properly handle internal predictors[12] since it assumes that their future values are independent of patient's survival and measured without error.[7] Importantly, it also assumes that the predictor has the same constant value between study-visits, until it suddenly changes when the next measurement is obtained (Figure 1A).[12] This assumption is unrealistic as we expect that biomarkers continuously change, and not only when measured. Consequently, TDCM would produce biased estimates of biomarkers' effect masking their true predictive ability. For example, Asar et al. studied whether repeatedly measured GFR_{Cr} predict initiation of RRT in 1611 patients from Chronic Renal Insufficiency Standards Implementation Study (CRISIS). They showed that the hazard ratios (HRs) for RRT were considerably underestimated by TDCM as compared to JMs (HRs per log-unit GFR_{Cr} decrease: 12.3 versus 38.7).[5] This advantage of JMs over TDCM has been demonstrated by

theoretical work and other simulation studies.[7,11-13]

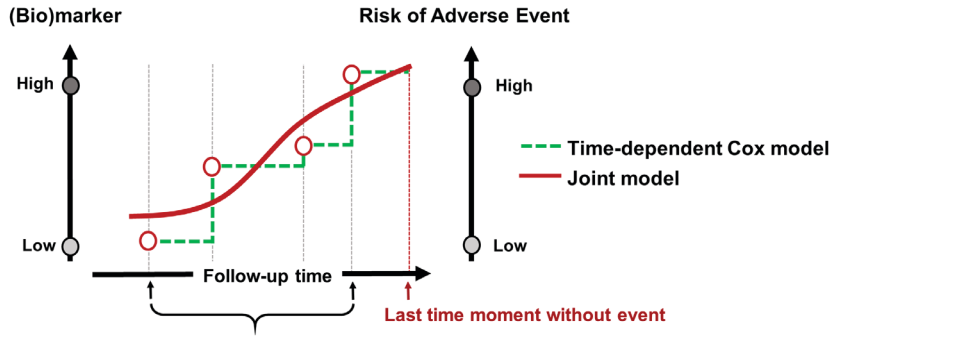
Methodological concept

The JMs combine two models: linear mixed-effects (LME) models and basic Cox model.[9] The LME models estimate a marker's trajectory using repeated measurements; Cox model estimates patients' time-to-event.

The LME models use the 2-component equation. The first "fixed-effect" component estimates a marker's average trajectory over all patients. The second "random-effect" component estimates by how much an individual patient deviates from this average trajectory (Figure 1B). By using these two components of information the patient-specific trajectory is constructed. Through the "random-effects" component they allow repeated measurements taken on the same patient to be correlated, and work well with unbalanced data.[12] Notably, the functional form of time is an important aspect of LME models. That is, in case the patient-specific trajectories are nonlinear, care should be given in the specification of the fixed- and random-effects components; polynomials or splines could be used to model such nonlinear profiles. Altogether, this allows a longitudinal trajectory estimated by LME models to correspond more naturally to the marker's biological evolution than the "jerkily" trajectory assumed by TDCM (Figure 1A).

Subsequently, JMs combine LME and Cox models to relate patient-specific trajectory to his/her prognosis (Figure S1). By doing this, JMs handles marker's missing data and measurement error that can occur during follow-up.[14] JMs are also advantageous when extreme values are observed because they postulate that the underlying rather than the observed value of the longitudinal biomarker is associated with the risk of an adverse endpoint (Figure 1A).

The basic assumptions behind LME and Cox models are the same as when they are separately analyzed. For continuous longitudinal data, we assume normally distributed error terms. The LME models also assume that discontinuation of the data-collection process for reasons other than the occurrence of the adverse endpoint are missing at random, i.e., these reasons can depend on covariates and past observed longitudinal values. For the endpoint a relative risk model is used with the proportional hazards assumption. Further reading on methodology[9], sample size and power determination[15] is provided elsewhere. Finally, JMs have been successfully applied for several medical conditions including HF, aortic aneurisms, aortic stenosis, heart, lung and kidney transplantation.[6,16-20]



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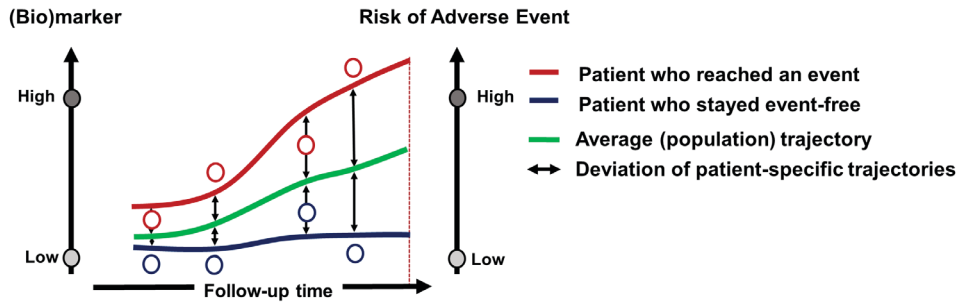

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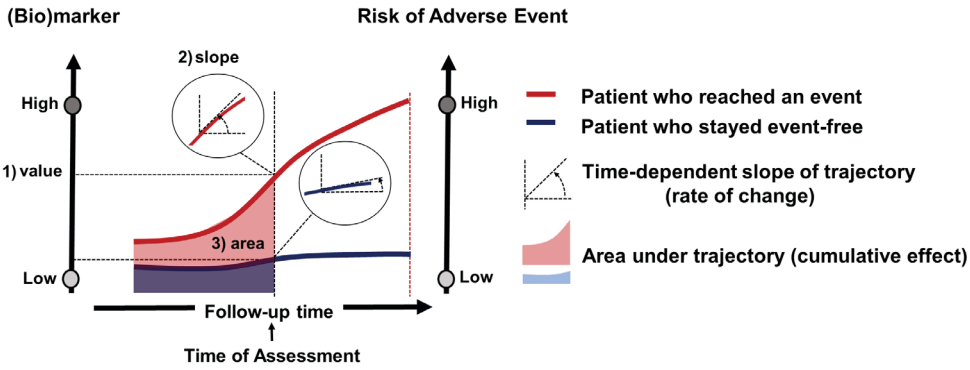

C

FIGURE 1 Graphical depiction of the difference between the marker's trajectory estimated by the time-dependent Cox model and the joint models and of the different aspects of time-varying markers. The X-axis displays follow-up time, the left Y-axis displays the value of a (bio)marker, and the right Y-axis displays a patient's risk prognosis. **Panel A** illustrates the marker's trajectories estimated by the time-dependent Cox model (green dashed line) and by the joint models (smooth red solid line) in the same patient. The panel shows that in the JMs the underlying profile represented by the red solid line is include in the relative risk model, and not the directly observed

value represented by the red circles which is what the Cox model does. In this way, JMs are advantageous because they account for the biological variation that the biomarker exhibits, but also in the settings when extreme values are observed but are not particularly helpful clinically (e.g., extremely low blood pressure). Interpretation of HRs from the JMs is the same as from the Cox model. **Panel B** illustrates how the patient-specific marker trajectory is constructed using linear mixed-effects models. The solid green line depicts the marker's value averaged over all patients at each of the study visits during follow-up (fixed-effect part), and the black arrows depict the deviation of the patient-specific values from the average values at the same study visits. Patient-specific trajectories are depicted for a patient who experienced the event (solid red line) and the one who did not (solid blue line). **Panel C** illustrates different aspects of time-varying markers that can be assessed by joint models: 1) marker's level, 2) slope of the marker's trajectory (rate of change), 3) area under the marker's trajectory (the cumulative effect of the marker's values). The time-dependent slope mathematically corresponds to the first derivative of the trajectory and the cumulative effect to the integral of the trajectory.

Components of time-varying markers

JMs tailor a patient's prognosis based on his/her own marker's values (Figure 1C). However, other components of the longitudinal marker can also be investigated.[7] For example, the rate at which a marker changes can be determined by estimating the instantaneous slope of its trajectory. The slope indicates by how much marker's values have been increasing or decreasing at the certain timepoint.[7] Consequently, disease's progression can be adequately quantified and related to prognosis. JMs can also assess entire history of marker values by estimating the area under its trajectory. The area indicates the cumulative effect of all values that the marker has taken up to the certain timepoint.[12] Altogether, JMs analyse comprehensively disease's dynamics to accurately profile patient's prognosis, wherein the application of TDCM is limited.

Personalized dynamic risk assessment

Patients are often seen in different disease's stages, react differently to treatment, or have other characteristics relevant for their phenotype. Thus, it is clear that a disease can differ both between patients and within the same patient over time. Consequently, a true marker's potential in ascertaining disease's severity in an individual, and its accurate relation to prognosis can only be revealed if individual (i.e., patient-specific) values are considered. For physicians, it is also medically relevant to utilize all available information (baseline and follow-up) to accurately detect disease's progression and profile better individual prognosis. JMs can easily update the patient's prognosis whenever additional information is collected, thereby assessing the risk in real-time.[14]

CONCLUSION

Although attention should be taken when analyzing repeatedly measured data, repeated-measures designs are valuable when assessing the dynamics of medical conditions. The use of JMs may improve patients monitoring by providing personalized dynamic risk predictions.

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SUPPLEMETARY INFORMATION

R code to fit joint model

Joint model will be fit using primary biliary cirrhosis (PBC) data collected at the Mayo Clinic from 1974 to 1984[1] available with the package JMBayes.[2] For the analysis we will consider 312 patients who have been randomized to D-penicillamine treatment and 154 patient randomized to placebo. During follow-up, serum bilirubin was collected on average 6 times per patient with a total of 1945 measurements. To assess how longitudinal trajectory of serum bilirubin relates to a patient-specific prognosis we have to use two datasets.

The first dataset is denoted by "**pbc2"** and contains repeatedly measured data organized in the long format (i.e., contains several rows per each patient; number of rows depends on how many samples the patient had provided). This dataset will be used to estimate longitudinal trajectory of serum bilirubin using linear mixed-effects (LME) models.

The second dataset is denoted by **"pbc2.id"** and contains patients' survival times organized in the wide format (i.e., contains a single row per patient). This dataset will be used to fit basic Cox model.

Full description of R codes provided below is discussed in the paper under reference 2.

```
# R code:
# first load package "JMbayes" and define the indicator "status2" as the
# composite event
# of transplantation or death

library("JMbayes")
pbc2$status2 <- as.numeric(pbc2$status != "alive")
pbc2.id$status2 <- as.numeric(pbc2.id$status != "alive")

# now fit the LME model
# variable "log(serBilir)" denotes logarithmically transformed marker: serum bilirubin
# variable "year" denotes the time from baseline when the marker was collected
# in this example, we used natural splines with two knots to better estimate marker's
# trajectory

lmeFit <- lme(log(serBilir) ~ ns(year, 2), data = pbc2,
random = ~ ns(year, 2) | id)
```

```
# now fit basic Cox model
# variable "years" denotes the time to event or censoring (note: this is different than variable
# "years" used for LME model)
# variable "status2" is event indicator
# variable "drug" denotes if a patient was randomized to D-penicillamine or placebo
# variable "age" denotes a patient's age at baseline
coxFit <- coxph(Surv(years, status2) ~ drug + age, data =
pbc2.id, x = TRUE)
# now fit joint model for the marker's value
jointFit.value <- jointModelBayes(lmeFit, coxFit, timeVar =
"year", n.iter = 30000)
summary(jointFit.value)
# calculate hazard ratio with corresponding 95% confidence interval
exp(confint(jointFit.value, parm = "Event"))
# in the output "Assoct" denotes HR for the value of log(serBilir)
# now fit joint model for marker's value and slope
dForm <- list(fixed = ~ 0 + dns(year, 2), random = ~ 0 +
dns(year, 2), indFixed = 2:3, indRandom = 2:3)
jointFit.value.slope <- update(jointFit.value, param = "td-
both", extraForm = dForm)
summary(jointFit.value.slope)
# calculate hazard ratio with corresponding 95% confidence interval
exp(confint(jointFit.value.slope, parm = "Event"))
# in the output "Assoct" denotes HR for the value of log(serBilir)
# in the output "AssoctE" denotes HR for the slope i.e., delta-log(serBilir)/year)
# the time-dependent slope mathematically corresponds to the first derivative of the
# trajectory
# now fit joint model for marker's cumulative effect
iForm <- list(fixed = ~ 0 + year + ins(year, 2), random = ~
0 + year + ins(year, 2), indFixed = 1:3, indRandom = 1:3)
jointFit.area <- update(jointFit.value, param = "td-extra",
extraForm = iForm)
summary(jointFit.area)
# calculate hazard ratio with corresponding 95% confidence interval
exp(confint(jointFit.area, parm = "Event"))
# in the output "AssoctE" denotes HR for the area under log(serBilir) trajectory
# the area mathematically corresponds to the integral of the trajectory
# Plotting marker's trajectory with corresponding survival probability in an
# individual patient
# in the following example we plotted serum bilirubin for patient number 4 from
# PBC data with survival
```

```
# probability for serum bilirubin value
ND <- pbc2[pbc2$id == 4, ]
sfit <- survfitJM(jointFit.value, newdata = ND)
plot(sfit, estimator = "mean", include.y = TRUE,conf.int =
TRUE, fill.area = TRUE, col.area = "lightgrey")
```

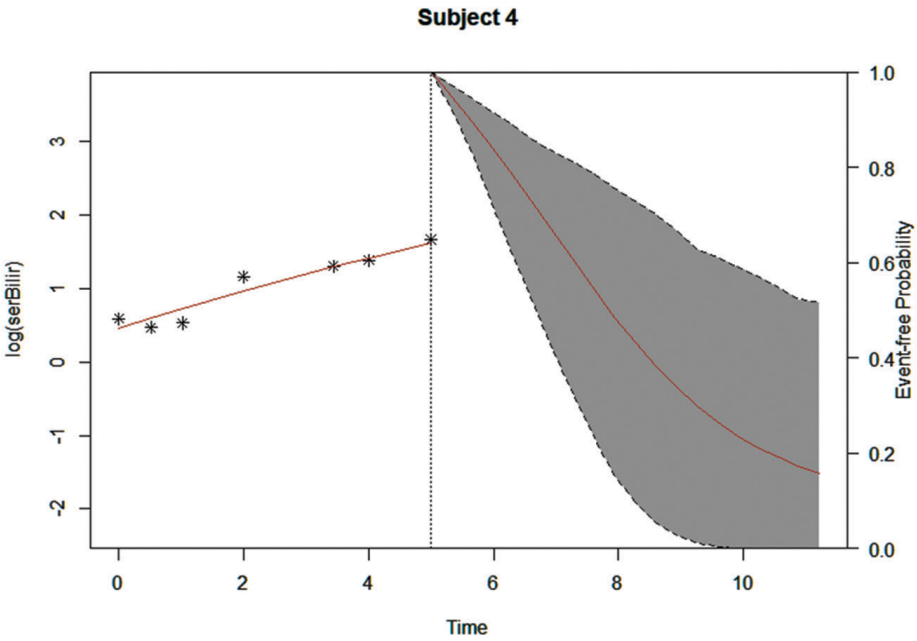

FIGURE S1 Personalized dynamic risk assessment using patient-specific trajectory of serum bilirubin. Serum bilirubin levels (on a log scale) are displayed on the primary (left) Y-axis and survival probability on the secondary (right) Y-axis. Follow-up time (years) is displayed on the X-axis. Patient-specific marker's trajectory (solid red line) with scatter points (asterisks) is displayed left of the vertical dotted black line. To the right of this line, the corresponding conditional survival probability curve (solid red line) is displayed with 95% confidence intervals (grey area).

Supplementary references:

1. Murtaugh PA, Dickson ER, Van Dam GM, Malinchoc M, Grambsch PM, Langworthy AL and Gips CH. Primary biliary cirrhosis: prediction of short-term survival based on repeated patient visits. *Hepatology (Baltimore, Md)*. 1994;20:126-34.
2. Rizopoulos D. The R Package JMbayes for Fitting Joint Models for Longitudinal and Time-to-Event Data Using MCMC. *Journal of Statistical Software*. 2016;72:46.

CHAPTER 3

On the Understanding of Statistical Interaction for Clinical Investigators

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Submitted

ABSTRACT

Despite testing for statistical interactions is usually stated as the secondary study objectives, it is not uncommon that these results lead to changing of treatment protocols or even modify the public health policies. For this reason, statistical interactions are studied frequently in clinical studies, but recent reviews have indicated that their proper assessment and reporting remains challenging for the clinical investigators. This article provides an overview of the challenges associated with the statistical interaction analysis to help the clinical investigators finding the best strategy to properly obtain and critically evaluate its presence in statistical models. Specifically, we discuss the importance of understanding the distinction between effect-measure modification and causal interaction, their qualitative and quantitative forms, the importance of a measurement scale on which interactions are tested, additive and multiplicative interaction measures, the relevance of multiple testing, and distinction between prespecified versus post-hoc analyses. Finally, we provide the recommendations that, if adhered to, could increase the clarity and the completeness of future studies. The understanding of the elements underlying statistical interaction analysis followed by its proper assessment and reporting may help in making the results more reliable, but also in facilitating clinical studies to use this type of analysis even more in the future.

INTRODUCTION

Many reasons motivate the study of statistical interaction of which the most fundamental are those to learn how to use an intervention most effectively, who would and who would not benefit (and who would benefit the most), or whether it would be harmful in specific subpopulations.[1] Although these reasons are usually stated as the secondary study objectives, if incorrectly performed statistical interaction analysis may cause false conclusions leading to unnecessary withholding of treatment, ineffective or even harmful treatment's effect.[2]

Despite the concept of statistical interaction is not new, it still poses a problem for the clinical investigators. In 2000, Assmann[3] et al. reviewed 50 randomized clinical trials (RCTs) in high-impact journals, and found that 70% of these trials performed interaction analysis but only 43% reported the test and 37% only a p-value. In 2006, Hernandez[4] et al. reported similar results after investigating published cardiovascular RCTs. In 2007, Wang[1] et al. evaluated 97 RCTs of which 61% used interaction analysis. Of those, 68% were unclear whether analyses were prespecified or post-hoc and only 27% reported an interaction test. Besides in RCTs, Knol[5] et al. found that vast majority of cohort and case-control studies also performed inappropriate interaction analysis. Finally in 2017, Wallach[6] et al. concluded that 61% of the RCTs the claimed the subgroup heterogeneity already in their abstracts (assuming these are the most credible) were, in fact, not supported by their results. For these reasons, previous reports tried to address this important topic.[1-3,7,8] These attempts, although informative, were directed for the most part to a narrow set of issues. For example, no discussion was performed for distinguishing different types of statistical interaction, or the importance of a measurement scale on which an interaction is tested. To date, a few reports[9,10] provide recommendations on some of these issues, but are intended mainly for an epidemiological audience.

In this paper, we summarize the evidence from the literature and provide the recommendations to assist the clinical investigators in selecting the best strategy to appropriately use, but also to critically evaluate, statistical interaction analyses as they might affect their decisions in clinical practice. In the following sections, we start by distinguishing different types and forms of statistical interaction; we then discuss how to properly analyze statistical interactions by the stratification or by an interaction modeling (i.e., inclusion of a cross-product term) and eventually how to report obtained results.

Types of statistical interaction

Statistical interaction can be classified as being either effect-measure modification or causal interaction. *Effect-measure modification* is present when the effect of one factor, exposure or intervention, on an outcome varies across the levels of another factor when no bias is present (Box 1).[11] Notably, the second factor does not need to affect the outcome for the effect-measure modification to be present, but only be related to another variable that does.[12] Some authors refer to this phenomenon also as an "effect heterogeneity".[13,14] Hence, the clinical motivation behind the effect-measure modification (or heterogeneity) analysis is to identify the subgroups of patients in whom a factor's effect differs based on patients' characteristics. If the effect of one factor is higher with higher levels of another factor an effect-measure modification is *positive*, whereas if this effect is lower an effect-measure modification is *negative*.

Causal interaction[15] is present when the combined effect of two factors on an outcome differs from their separate effects when no bias is present. (Box 1).[11] Unlike for effect-measure modification, both factors have to be causally related to an outcome in order for causal interaction to be present.[16] Despite it sounds theoretical, this distinction is important to be made especially if an intervention on the secondary factor is of interest.[17] For example, if an investigator would like to test whether cholesterol-lowering drug reduces the risk of myocardial infarction, and a positive interaction between the cholesterol treatment and hypertension is observed this would indicate that hypertension modifies the treatment's effect. Thus, targeting the subgroup of patients with hypertension would maximized the treatment's effect. However, if an investigator would also be interested in testing whether introducing secondary intervention (i.e., antihypertensive treatment) would further reduce the risk of myocardial infarction he/she should make sure that the secondary factor (i.e., hypertension) not only modifies the effect of the cholesterol treatment but is causally related to myocardial infarction. If so, causal interaction is present and a factorial design can be applied to confirm the hypothesis. Finally, a positive causal interaction indicates that the effect of two factors together is larger than the two factors considered separately, whereas a negative causal interaction indicates that this joint effect is smaller than these effects considered separately.

Effect-measure modification

- To assess if the effect of a factor (i.e., exposure or intervention) varies across levels of another factor when no bias is present
- Synonyms: effect modification, effect heterogeneity
- It can be positive or negative; qualitative or quantitative

Causal interaction

- To assess if the combined effect of two factors together (i.e., exposures or interventions) is different than their separate effects when no bias is present
- It can be positive or negative; qualitative or quantitative

BOX 1 Types and forms of statistical interaction. In a concrete analysis, the term "effect-measure" should be replaced with the name of exact measure that is used to estimate the effects in the statistical model. For example, if one would use the logistic regression model, a statistical interaction should be reported as the odds-ratio modification (or heterogeneity). Similarly, if Cox regression model is applied then hazard-ratio modification (or heterogeneity) would be more appropriate terminology. In this way, ambiguity about which effect is tested would be resolved.

Forms of statistical interaction

Statistical interaction can take either quantitative or qualitative form. The *quantitative* form (synonym[18]: "non-crossover") is the most common and is present when an effect of one factor has a different magnitude, but in the same direction, across strata of another factor (Figure 1: 1-4, 7, and 8).

The *qualitative* form (synonym[18]: "crossover") is present (1) if one factor does not have an effect on the outcome in one stratum, but does have effect in other stratum, of the second factor (Figure 1: 5a and 6a) or (2) if one factor has opposite effects depending on the strata of the second factor (Figure 1: 5b and 6b). Of note is that detection of qualitative interactions also depends on a study's selection criteria. For example, angiotensin-converting-enzyme inhibitors are beneficial in hypertensive patients, but are harmful in hypertensive patients due to reno-vascular disease.[19] If the latter group is excluded from the study due to selection criteria, an important qualitative interaction will be missed. This may lead to serious consequences if the study concludes that both groups of patients should be treated identically.

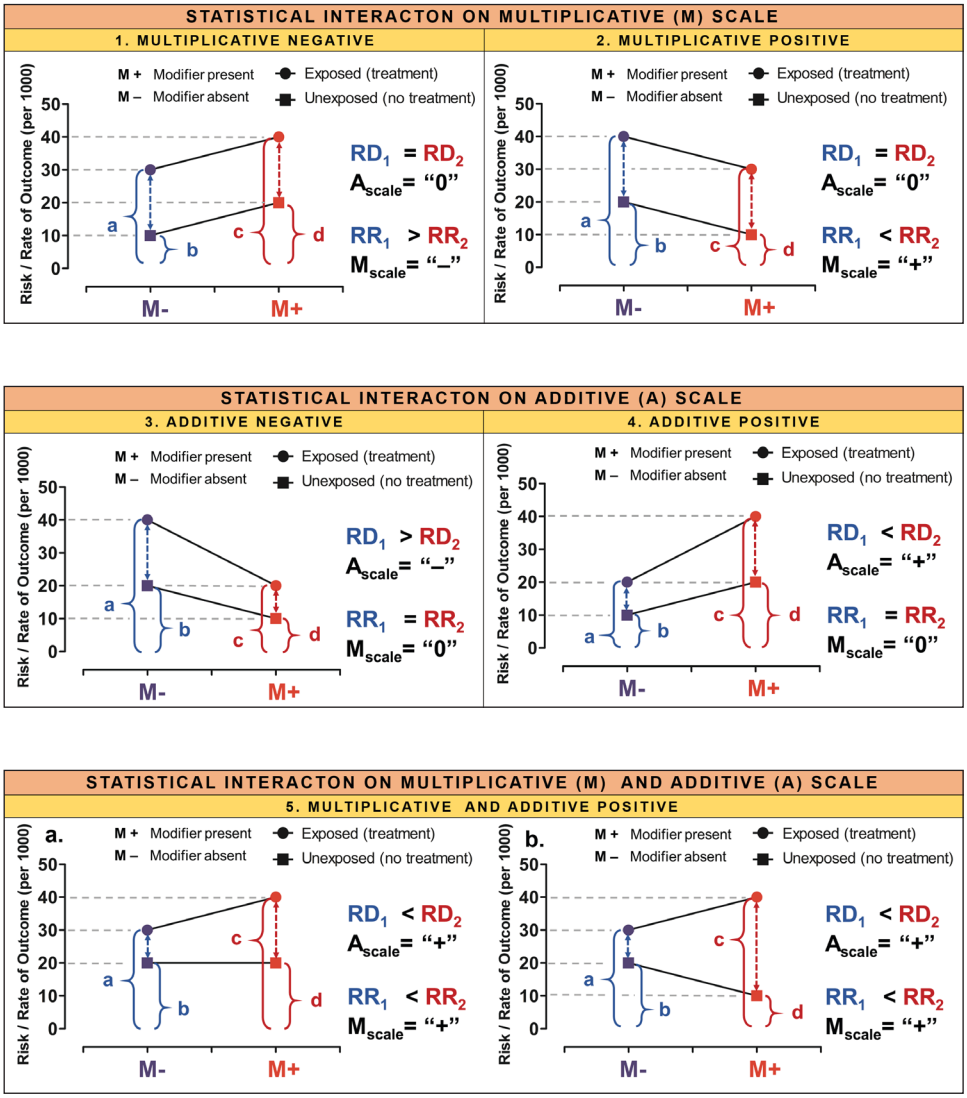
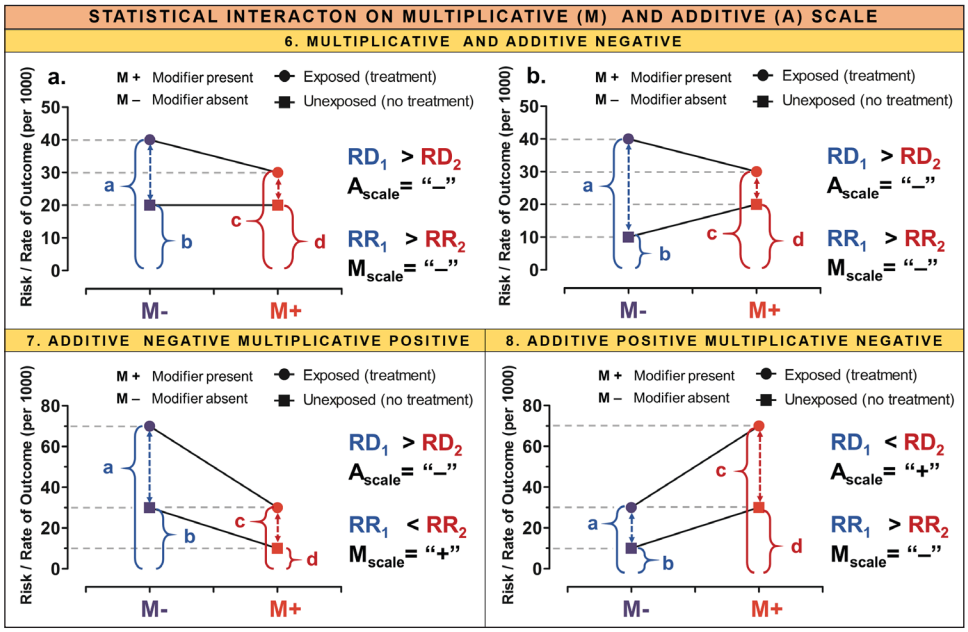


FIGURE 1 Potential scenarios that can be found when statistical interaction is detected by additive and multiplicative scales simultaneously. "a" denotes the effect in exposed (or treated) subgroup without modifier M; "b" denotes the effect in unexposed (or untreated) subgroup without modifier M; "c" denotes the effect in exposed (or treated) subgroup with modifier M; "d" the effect in unexposed (or untreated) subgroup with modifier M. RD_1 can be calculated as a – b; RR_1 can be calculated as a / b; RD_2 can be calculated as c – d; RR_2 can be calculated as c / d; numbers presented on Y-axes can be used to calculate RD_1, RD_2, RR_1, and RR_2. If there is departure on one of the two scales, eight possible scenarios can be observed: 1) no additive departure ($RD_1 = RD_2$), but negative multiplicative departure ($RR_1 > RR_2$); 2) no additive departure ($RD_1 = RD_2$), but positive multiplicative departure ($RR_1 < RR_2$); 3) no multiplicative departure ($RR_1 = RR_2$), but negative additive departure ($RD_1 > RD_2$); 4) no multiplicative departure ($RR_1 = RR_2$), but positive additive departure ($RD_1 < RD_2$); 5)



positive multiplicative and additive departures ($RD_1 < RD_2$ and $RR_1 < RR_2$) with two additional situations 5a) the effect is present only in one subgroup or 5b) the opposite effects are present in subgroups; 6) negative multiplicative and additive departures ($RD_1 > RD_2$ and $RR_1 > RR_2$) with two additional situations 6a) the effect is present only in one subgroup or 6b) the opposite effects are present in subgroups; 7) negative additive ($RD_1 > RD_2$) and positive multiplicative departures $RR_1 < RR_2$); 8) positive additive ($RD_1 < RD_2$) and negative $RR_1 > RR_2$) multiplicative departures.

ASSESSMENT OF STATISTICAL INTERACTION

As noted above, there are two ways to assess statistical interactions: (1) *stratification* (i.e., stratified or subgroup analysis) in which the effect of one factor is assessed within strata of another factor separately, (2) *interaction modeling* in which both factors are included into a statistical model together with their cross-product term ($F_1+F_2+F_1{}^*F_2$).

Before introducing their technical descriptions it is important to note that a statistical interaction is observed only if there is a departure from an underlying measurement scale on which a statistical model estimates effects. This means that a statistical interaction is scale-dependent. However, different statistical models estimate effects on different measurement scales. For example, standard linear regression coefficients estimate the sum of effects on an additive scale, whereas standard logistic regression and Cox regression exponentiated coefficients estimate the product of effects on a multiplicative scale such as risk ratio (RR), odds ratio (OR), or hazard ratio (HR)

scale. Importantly, additive and multiplicative scales do not always provide us with the same conclusion whether a statistical interaction is present or in which direction it operates. For this reason, both additive and multiplicative interaction measures are discussed below.

Additive interaction measures

A departure on an additive scale would mean that the combined effect of two factors is larger (in case of positive interaction) or smaller (in case of negative interaction) than the sum of their individual effects.[20]

For a binary outcome, e.g., death ("yes", "no"), and two binary factors, e.g., disease A and disease B ("yes", "no"), an additive interaction can be assessed using stratification and expressed as the absolute excess risk due to interaction (AERI) (Table 1: equation-1). For example, Weiner et al. studied the effects of chronic kidney disease (CKD) and cardiovascular disease (CVD) on the 10-year risk of the composite endpoint including cardiovascular and all-cause death.[21] Authors reported the absolute cumulative risk of 66% in individuals with both CKD and CVD, 34% in those with CKD but without CVD, 38% in those without CKD but with CVD, and 15% in those without CKD or CVD. The AERI is calculated as 66 + 15 – 34 – 38 = 9% which indicates a super-additive (i.e., positive) interaction because AERI >0 (detailed calculations are described in the supplemental text). This also indicates an absolute excess risk of 9% due to the interaction itself.

For a continuous outcome (e.g., blood pressure), and two categorical or continuous factors or their combination, an additive interaction can be assessed by including both factors together with their cross-product term into a linear regression model (Table 1: equation-2). In this case, β coefficient for the cross-product term would quantify the interaction on an additive scale.

When using continuous factors, a magnitude of statistical interaction will differ based on its unit-scale.[20] For example, if an investigator assesses whether a patient's age modifies the treatment's effect, the magnitude of the interaction between age and treatment will differ if age is expressed per 1-year, 5-year interval, or in some other units. Finally, a nice feature of regression models is that controlling for other covariates can easily be performed by including them into the model.

Multiplicative interaction measures

A departure on a multiplicative scale would mean that the combined effect of two factors is larger (in case of positive interaction) or smaller (in case of negative interaction) than the product of their individual effects.[20] Thus, the multiplicative scale corresponds to the ratios of effects rather than their difference as the additive scale does.

For a binary outcome and two binary factors, a multiplicative interaction can be assessed using stratification and expressed as the ratio of RRs (Table 2: equation-11). In the example above[21], the RRs of composite endpoint were 4.4 in individuals with both CKD and CVD, 2.3 in those with CKD but without CVD, 2.5 in those without CKD but with CVD as compared to those with neither, and 1.0 in those without CKD or CVD (supplemental text). Here, a multiplicative interaction is calculated as 4.4 / (2.5 * 2.3) = 0.8 which indicates a sub-multiplicative (i.e., negative) interaction between CKD and CVD because the ratio of RRs <1. This also indicates relative risk ratio due to interaction of -20%. However, the AERI indicated their super-additive interaction with absolute excess risk of 9%. Therefore, this example illustrates an aforementioned point that a measurement scale influences the presents and the direction of a statistical interaction.

For a binary outcome and two categorical or continuous factors or their combination, a multiplicative interaction can be assessed by including both factors together with their cross-product term into the logistic or Cox regression model (Table 2: equation-12 and equation-13). In the example above[21], OR or HR for the cross-product term would correspond to 0.8 indicating a sub-multiplicative interaction.

Additive versus Multiplicative scale

Figure 1 illustrates eight potential scenarios that can be found when statistical interaction is detected by additive and multiplicative scales simultaneously. In six of eight scenarios (Figure 1: 1-4, 7, and 8) these scales carry different information regarding statistical interaction. Therefore, it is not only possible, but even common to come to the different conclusions depending on the scale on which a statistical interaction is tested.

From the public health perspective, several authors have argued that under assumption that benefits, or costs, of certain factors are measured by excess, or reduction, in incident numbers (i.e., case-load per unit population), additive measures are more reliable than multiplicative measures to increase a net benefit by targeting the proper subpopulation.[13,22] The main reasoning behind was that if an excess effect

produced by each factor is nonadditive, a public health impact can only be predicted if the levels of all factor are known.[23,24]

Another important point is that both interaction measures can be considerably affected by falsely negative results, i.e., a type 2 error. This is because studies are usually only powered to show the significant differences in the total cohort and not in the subgroups.[3] In this context, obtaining significant p-values may be even more difficult when testing departure from additivity than from multiplicity of effects.

Taken together with previous reports,[9,16] we strongly advise the clinical investigators to report both additive and multiplicative interaction measures with corresponding 95% confidence interval (CI).

Additive interaction measures derived from multiplicative statistical models

Although statistical models such as logistic regression and Cox regression models operate on a multiplicative scale, additive interaction measures can still be calculated (Box 2). The following formulae apply for all ratio-measures (RR, OR, HR) equally.[16,25,26]

Relative Excess Risk due to Interaction (RERI)

The RERI (synonym: interaction contrast ratio [ICR]) is the difference between joint relative effect of two factors and their relative effects considered separately (Table 1: equations-3 and equations-4).[13] Although RERI is an additive interaction measure, it differs from the AERI because it operates with ratios instead of absolute risks. However, when only ratio-measures are given, the RERI can be used to determine additive interaction effect. For example, Jorgensen et al. reported that the 30-day risk of major adverse cardiovascular events (MACE) was associated with long-term use of β-blockers in patients with uncomplicated hypertension undergoing non-cardiac surgery.[27] They also found a super-multiplicative interaction between β-blocker use and diabetes. To quantify this interaction on an additive scale, we calculate the RERI using equation-3 as 2.20 –1.47 – 0.94 + 1.00 = 0.79 (supplemental text). The RERI indicated a super-additive interaction between β-blocker and diabetes (RERI >0). The 95%CI for RERI can be calculated using the delta method[28] or using the first percentile Bootstrap method which covers 95%CI better than the delta method[29] and is more suitable for continuous factors.[20] An interpretation of RERI may be sometimes less straightforward if additional covariates are included in the model because it varies across the levels defined by additional covariates.[30] The codes for calculating RERI with 95%CI are available in SAS[12,25,31], STATA[12], R[32,33], or using excel sheets.[9,20]

Relative Excess Risk due to Interaction (RERI)

- Use RR, OR, and HR
- RERI > 0, super-additive statistical interaction
- RERI < 0, sub-additive statistical interaction
- Depends on additional covariates adjustment

Attributable proportion due to interaction (AP)

- Use RR, OR, and HR
- Same direction as RERI
- Proportion of the outcome in double exposed group that is due to the interaction itself
- Depends on additional covariates adjustment

Modified AP*

- Use RR, OR, and HR
- Same direction as RERI
- Proportion of the joint effect of both exposures that is due to the interaction itself
- Does not depend on additional covariates adjustment

Synergy (S)-index

- Use RR, OR, and HR
- S-index > 1, super-additive statistical interaction
- S-index < 1, sub-additive statistical interaction
- Does not depend on additional covariates adjustment
- Interpretation is difficult if one or both factors are preventive

BOX 2 Additive interaction measures derived from the multiplicative (log-linear, logistic, Cox regression) models. RR, risk ratio; OR, odds ratio; HR, hazard ratio.

Attributable proportion due to interaction (AP)

The attributable proportion for the outcome, denoted here by AP, indicates the proportion of the outcome in double exposed group that is due to the interaction itself.[34] It is derived from RERI (Table 1: equation-5 and equation-6). Following the above example by Jorgensen[27], we calculate AP using equation-5 as 0.79 / 2.2 = 0.36 indicating that 36% of MACE in patients with diabetes and on β-blockers is due to the interaction itself. Similar to RERI, AP varies if additional covariates are included into the model. The codes for calculating AP with 95%CI are available in SAS[12,25,31], R[32], or using excel sheets.[9,20]

Alternatively, the attributable proportion for the effects, denote here by AP*, can be calculated which represents the proportion of the joint effect of both exposures that is due to the interaction itself (Table 1: equations-7 and equations-8).[34] In the same example[27], AP* can be calculated using equation-7 as 0.79 / (2.2 – 1) = 0.66 which indicates that 66% of joint effect of diabetes and β-blockers use is due to the interaction itself. Notably, AP* is independent of covariates adjustment.[34] The codes for calculating AP* with 95%CI are available in SAS[35], STATA[35], and R.[32,33]

TABLE 1 Additive measures of statistical interaction.

A. From additive statistical models:	**Eq. n.**
Absolute excess risk due to interaction (AERI) (using stratification)	
Formula: $AERI = R_{E+,M+} + R_{E-,M-} - R_{E+,M-} - R_{E-,M+}$ Description: E, the exposure (i.e., primary factor); M, a modifier (i.e., secondary factor); $R_{E+,M+}$, the risk in the patients who are exposed to both factors; $R_{E-,M-}$, the risk in the patients in whom both factors are absent; $R_{E+,M-}$, the risk in the patients who are exposed only to the primary factor; $R_{E-,M+}$, the risk in the patients who are exposed only to the secondary factor.	(1)
Linear regression model (using a cross-product term)	
Formula: $Y\ (continuos) = \beta_0 + \beta_1(E) + \beta_2(M) + \beta_3(ExM)$ Description: β_0, average Y in patients in whom both factors are absent (E–, M–); β_1, average difference in Y between the patients who are exposed only to the primary factor (E+, M–) and those in whom both factors are absent (E–, M–); β_2, average difference in Y between the patients who are exposed only to the secondary factor (E–, M+) and those in whom both factors are absent (E–, M–); $\beta_1+\beta_2+\beta_3$, average difference in Y between the patients in whom both factors are present (E+, M+) and those in whom both factors are absent (E–, M–); β_3, a coefficient for the cross-product term that represents an additive interaction measure.	(2)

continued

B. From multiplicative statistical models:	**Eq. n.**
Relative excess risk due to interaction (RERI)	
Formulae (can be used for RR, OR, HR equally): $RERI_{RR} = RR_{E+,M+} - RR_{E+,M-} - RR_{E-,M+} + 1$ (using stratification) $RERI_{OR} = OR_E \times OR_M \times OR_{ExM} - OR_E - OR_M + 1$ (using a cross-product term) Description: $OR_E \times OR_M \times OR_{ExM}$ equals to $OR_{E+,M+}$. Note: $OR_{E+,M+}$ is not provided in the output of the regression models using a cross-product term. The RERI is the difference between joint relative effect of two factors and their effects considered separately.	(3) (4)
Attributable proportion due to interaction (AP)	
Formulae (can be used for RR, OR, HR equally): $AP = RERI_{RR} / RR_{E+,M+}$ (using stratification) $AP = RERI_{OR} / (OR_E \times OR_M \times OR_{ExM})$ (using a cross-product term) Description: The AP is the proportion of the outcome in double exposed group that is due to the interaction itself.	(5) (6)
Modified attributable proportion due to interaction (AP*)	
Formulae (can be used for RR, OR, HR equally): $AP^* = RERI_{RR} / (RR_{E+,M+} - 1)$ (using stratification) $AP^* = RERI_{OR} / (OR_E \times OR_M \times OR_{ExM} - 1)$ (using a cross-product term) Description: The AP* represents the proportion of the effect of both exposures due to the interaction itself.	(7) (8)
Synergy (S)-index	
Formulae (can be used for RR, OR, HR equally): $S = (RR_{E+,M+} - 1) / [(RR_{E+,M-} - 1) + (RR_{E-,M+} - 1)]$ (using stratification) $S = (OR_E \times OR_M \times OR_{ExM} - 1) / [(OR_E - 1) + (OR_M - 1)]$ (using a cross-product term) Description: The S-index is the extent to which joint relative effect of two factors together exceed 1, and whether this exceeding is greater than the sum of relative effects of two factors separately exceed 1.	(9) (10)

Eg. n., equation number.

Synergy index

The S-index reflects the extent to which the joint relative effect of two factors together exceed 1, and whether this exceeding is greater than the sum of relative effects of two factors separately exceed 1 (Table 1: equation-9 and equation-10). For example, Andrews et al. studied the effect of an early resuscitation protocol on the in-hospital mortality in septic patients with hypotension.[36] They found that the use

of early resuscitation protocol increased the in-hospital mortality which was more pronounced in patients with Glasgov coma scale score (GCS) 13-15 than in those with score 3-12. The S-index can be calculated using equaiton-9 as (3.55 – 1) / (3.09 – 1 + 1.91 – 1) = 0.85 indicating a sub-additive interaction between the treatment protocol and worse GSC score because S-index <1 (supplemental text). Notably, the S-index is in independent of covariates adjustment.[30] However, the interpretation may be difficult if one of the factors are preventive rather than causative, i.e., when denominator of S-index is negative.[37] The codes for calculating S-index with 95%CI are available in SAS[12,25,31], R[33], or using excel sheets.[9,20]

TABLE 2 Multiplicative measures of statistical interaction.

Relative risk ratio due to interaction (using stratification)	**Eq. n.**
Formulae (can be used for RR, OR, HR equally): $RR_{E+,M+} / (RR_{E+,M-} \times RR_{E-,M+})$ Description: $RR_{E+,M+} / (RR_{E+,M-} \times RR_{E-,M+})$ equals to the relative risk of a product term in a regression model	(11)
Logistic regression model (using a cross-product term)	
Formula: $Ln[Pr_{Y=1}/(1 - Pr_{Y=1})] = \beta_0 + \beta_1(E) + \beta_2(M) + \beta_3(ExM)$ (exponentiation of both sides of equation to eliminate logarithm) $Pr_{Y=1} / (1 - Pr_{Y=1}) = e^{\beta 0} \times e^{\beta 1(E)} \times e^{\beta 2(M)} \times e^{\beta 3(ExM)}$ (this can also be rewritten as) $Odds = O_0 \times OR_E \times OR_M \times OR_{ExM}$ Description: O_0, odds of Y=1 (e.g., a patient dies) in patients in whom both factors are absent (E–, M–) i.e., this is a background risk because odds of outcome are determined by factors other than E and M; OR_E, odds ratio between the patients who are exposed only to the primary factor (E+, M–) and those in whom both factors are absent (E–, M–); OR_M, odds ratio between the patients who are exposed only to the secondary factor (E–, M+) and those in whom both factors are absent (E–, M–); $OR_E \times OR_M \times OR_{ExM}$, odds ratio between the patients who are exposed to both factors together (E+, M+) and those in whom both factors are absent (E–, M–); OR_{ExM}, OR for the cross-product term, that represents a multiplicative interaction measure.	(12)
Cox regression model (using a cross-product term)	
Formula: $Ln[H(t)] = \beta_0 + \beta_1(E) + \beta_2(M) + \beta_3(ExM)$ $H(t) = e^{\beta 0} \times e^{\beta 1(E)} \times e^{\beta 2(M)} \times e^{\beta 3(ExM)}$ (this can also be rewritten as) $H(t) = H_0(t) \times HR_E \times HR_M \times HR_{ExM}$ Description: The same description as for logistic model, but hazard ratio are used instead of odds ratio. HR_{E+xM+}, HR for the product term that represents a multiplicative interaction measure.	(13)

Eg. n., equation number.

Multiple testing

Multiple testing is common problem when testing statistical interactions because different data, hypotheses, and analyses are assessed simultaneously. Figure 2 outlines four steps that should be considered to reduce the probability of the false positive results, i.e., type 1 error. In hypothesis-generating studies, some authors suggest that no adjustments of the p-value are required.[38] In hypothesis-confirmatory studies, an adjustment for multiple testing should be done as these studies often lead to policy-making. To date, several methods exist to address multiple testing and are described elsewhere.[38] Finally, a multiple testing represents another reason why forming conclusions solely based on the p-value of an interaction test is unjustified.

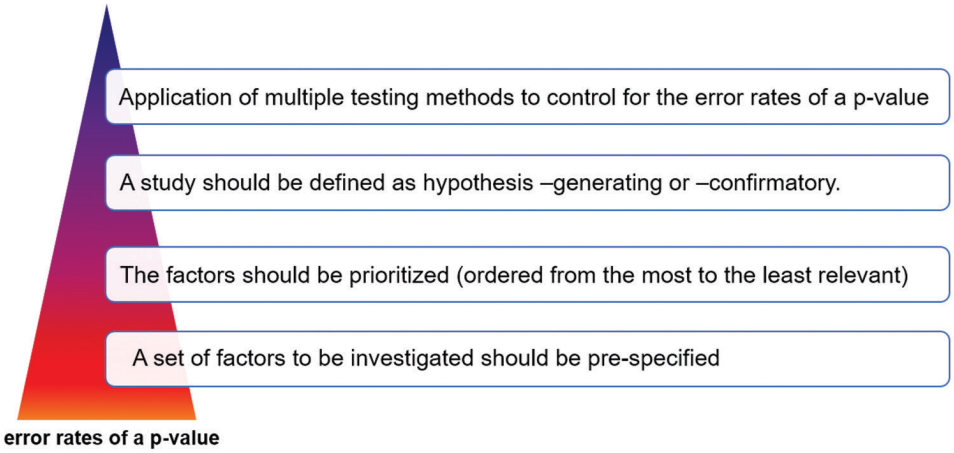


FIGURE 2 Four steps to be considered to reduce probability of having a significant interaction only as a result of chance findings.

Sample size calculation

Sample size calculation should be considered if an investigator is planning to analyze statistical interaction, and especially if an important subgroup analysis is expected to be performed. This helps defining the rule for stopping a trial in order for an adequate number of patients is recruited for each subgroup. For this purpose, a number of software programs[39] and excel sheets are available both for additive[40] and multiplicative[41] interaction measures and various study designs.[42]

REPORTING OF STATISTICAL INTERACTION

To make the results of an interaction analysis more reliable, an investigator should report all relevant information regarding the analysis which are discussed below.

The goal of statistical interaction analysis and set of confounders based on that goal (methods)

An important question that should be answered firstly is why statistical interaction is tested. For example, is the aim to find the subgroup of patients based on their baseline characteristics where the treatment has the greatest effect, or is intervening on those characteristics also considered? This is important to state because different set of confounders should be then chosen to control for the bias.

If effect-measure modification is investigated, only confounding of the primary factor on an outcome should be controlled for. In RCTs, this confounding of the treatment's effect is already addressed by randomization. Yet, one may still want to control for confounding in order to eliminate the possible imbalances between the subgroups that may occur despite the randomization.[17] However, if causal interaction is investigated, then confounding for the effects of both factors on an outcome must be controlled for.[17]

The origin of statistical interaction analysis (methods)

Based on the origin, statistical interaction can be classified as being either prespecified or post-hoc. The prespecified analysis[6] (synonyms: "a priory", "preplanned", "planned", "previously suggested") is considered if the analysis is specified before data are obtained. This specification includes: 1) factors that are considered for analysis, 2) outcomes that are considered for analysis, and 3) set of confounders. An investigator may also consider an attempt of corroboration, i.e., a subsequent study with the same analysis as reported previously (for the same strata, interventions, outcomes, and study population) as the prespecified analysis.[6]

The post-hoc analysis[6] (synonyms: "non-prespecified", "secondary", "explanatory", "preliminary") is considered in all other situations. Of note is that post-hoc analyses are usually data-driven and may be motivated with overall null findings.[43] In this case, one could aim to systematically assess all possible statistical interactions in order to reduce a chance of spurious results.[44] Nonetheless, the post-hoc analyses should be considered solely for exploratory purposes.

The results of statistical interaction analysis (results)

For effect-measure modification between two categorical factors, the results should include 1) effects per each stratum of both factors using a single reference category that should be a subgroup with the lowest risk, 2) effects of the primary factor in strata of the secondary factor, 3) effect per each multivariable adjusted models; 4) additive and multiplicative interaction measures with 95%CI; 5) the set of confounders for the primary factor–outcome relationship (template table 2). For causal interaction, the results should also include 6) effects of the secondary factor in strata of the primary factor; 7) the set of confounders for the secondary factor–outcome relationship (template table 2).

If one of the factors is continuous, a 2 x 2 table cannot be constructed and the results should be reported as in the template table 3. For easier interpretation, it is advisable to present the results using figures, which may also be helpful if more than two factors are tested. How these figures can be made in R is described elsewhere.[45] Alternatively, a continuous variable can be dichotomized and reported as in the template tables 1 and 2.

CONCLUSION

This article outlines the challenges associated with assessment and reporting of statistical interactions in clinical studies, as well as the recommendations that, if adhered to, could increase the clarity and the completeness of future studies. In the present article, we have discussed the importance of the distinction between effect-measure modification and causal interaction, their qualitative and quantitative forms, the importance of a measurement scale on which interactions are tested, additive and multiplicative interaction measures, the relevance of multiple testing as well as the origin of interaction analysis (i.e., whether is prespecified or post-hoc). In addition, we have summarized the information on publicly available SAS, STATA, and R codes, as well as the excel sheets, which can freely be used to calculate different interaction measures. Likewise, we have provided the templates to report obtained results. Altogether, we believe that this article will help in making the results of statistical interaction more reliable, and facilitate clinical studies to use this type of analysis even more in the future.

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SUPPLEMENTARY INFORMATION

Example 1: Calculation of the absolute excess risk due to interaction (AERI) in the section entitled Additive interaction measures.

Weiner et al. studied the effects of chronic kidney disease (CKD) and cardiovascular disease (CVD) on the 10-year risk of the composite endpoint including cardiovascular and all-cause death.[21] Using numbers provided in the Tables 1 and 2 of their article, we can calculate absolute cumulative 10-year risk per each subgroup as shown in the table below:

Absolute risks		**Cardiovascular disease (CVD)***	
		No	Yes
Chronic kidney disease (CKD)*	No	15% (3053/20970)	38% (1344/3519)
	Yes	34% (565/1664)	66% (501/759)

*In parenthesis is shown the number of patients with event divided by the total number of patients in the corresponding subgroup.

To calculate AERI we will use an equation-1: AERI = $R_{CVD+,CKD+}$ + $R_{CVD-,CKD-}$ – $R_{CVD+,\ CKD-}$ – $R_{CVD-,CKD+}$ and calculate as 66% +15% - 38% - 34% = 9%. The AERI indicates a super-additive interaction between CKD and CVD because AERI > 0, but also shows an absolute excess risk of 9% due to the interaction itself.

Calculation of the ratio of RRs in the section entitled Multiplicative interaction measures.

In the same study by Weiner et al.[21] we can further calculate relative risk ratio due to interaction as shown in the table below. In the following table, relative risks are calculated by dividing the absolute risks per each subgroup with the risk in the subgroup of patients without CVD or CKD, i.e, subgroup with the lowest absolute risk.

Relative risks		**Cardiovascular disease (CVD)***	
		No	Yes
Chronic kidney disease (CKD)*	No	1.0 (15%/15%)	2.5 (38%/15%)
	Yes	2.3 (34%/15%)	4.4 (66%/15%)

*In parenthesis is shown relative risk which is calculated by dividing the absolute risk in the subgroup with the risk in the subgroup of patients without CVD or CKD, i.e, subgroup with the lowest absolute risk.

To calculate ratio of risk ratios we will use equation-11: $RR_{CVD+,CKD+}$ / ($RR_{CVD+,\ CKD-}$ x $RR_{CVD-,CKD+}$) and calculate as 4.4 / (2.5 x 2.3) = 0.8. This indicates a sub-

multiplicative interaction between CKD and CVD because the ratio of RRs < 1, but also shows relative risk ratio due to interaction of -20%.

Example 2. Calculation of the relative excess risk due to interaction (RERI) in the section entitled Relative Excess Risk due to Interaction (RERI).

Jorgensen et al. studied the effect of the long-term β-blockers use on the the 30-day risk of major adverse cardiovascular events (MACE) in patients with uncomplicated hypertension undergoing non-cardiac surgery.[27] Authors found a multiplicative interaction between the long-term use of β-blockers and diabetes on the 30-day risk of MACE. Using numbers provided in Figure 3 of their article, we can calculate both AERI and RERI of the aforementioned interaction as shown in the table below:

Absolute risks		**Diabetes (DM)***	
		No	Yes
β-blockers use*	No	0.85% (294/34691)	0.80% (48/5985)
	Yes	1.25% (164/13096)	1.87% (29/1548)

*In parenthesis is shown the number of patients with event divided by the total number of patients in the corresponding subgroup.

To calculate AERI we will use an equation-1: AERI = $R_{\beta\text{-blockers+,DM+}} + R_{\beta\text{-blockers–,DM–}} – R_{\beta\text{-blockers+, DM–}} – R_{\beta\text{-blockers–,DM+}}$ and calculate as 1.87% + 0.85% - 1.25% - 0.80% = 0.67%. The AERI indicates a super-additive interaction between the long-term use of β-blockers and diabetes with an absolute excess risk of 0.67% due to the interaction itself.

To calculate RERI we will use an equation-3: $RERI_{RR} = RR_{\beta\text{-blockers+,DM+}} – RR_{\beta\text{-blockers+, DM–}} – RR_{\beta\text{-blockers–,DM+}} + 1$. Furthermore, to obtain the relative risks we will divide the absolute risks per each subgroup by 0.85% which is the risk in patients who did not take β-blockers and did not have diabetes. Thus, the calculation is as follows $RERI_{RR}$ = 1.87% / 0.85% - 1.25% / 0.85% - 0.80% / 0.85% +1 = 0.79 indicating a super-additive interaction because $RERI_{RR} > 0$. Note that, although both AERI and RERI shows additivity of interaction, they are not the same (0.67≠0.79). This is because AERI operates with on a risk-difference scale and relative risk-difference scale.

Calculation of Attributable proportion due to interaction (AP) and modified AP* in the section entitled Attributable proportion due to interaction (AP).

In the same study by Jorgensen et al.[27] we can extend our investigation by calculating the attributable proportions of the outcome and of the joint effect that are due to the interaction itself. For former calculation, we will use an equation-5: AP =

$RERI_{RR}$ / $RR_{\beta\text{-blockers+,DM+}}$ and calculate as 0.79 / (1.87% / 0.85%) = 0.36 indicating that 36% of the 30-day risk of MACE is due to the interaction itself. For latter calculation, we will use an equation-7: AP* = $RERI_{RR}$ / ($RR_{\beta\text{-blockers+,DM+}}$ - 1) and calculate as 0.79 / (1.87% / 0.85% -1) = 0.66 indicating that 66% of the joint effect of β-blockers and diabetes is due to interaction itself.

Example 3. Calculation synergy index (S-index) in the section entitled Synergy index.

Andrews et al. studied the effect of an early resuscitation protocol on the in-hospital mortality in septic patients with hypotension.[36] Authors found a multiplicative interaction between the intervention and patients baseline Glasgov coma scale score (GCS). In their article, GSC score was tested as ordinal variable with three categories ≥13, 12-9, and 8-3. Considering that effect in the latter two categories were similar we dichotomized GSC score into ≥13 and 12-3. Using numbers provided in Figure 3 of their article, we can calculate S-index as shown in the table below:

Absolute risks		**GSC score***	
		≥ 13	3-12
Treatment*	usual care	22% (17 / 78)	68% (15 / 22)[a]
	early resuscitation protocol	42% (36 / 86)	78% (14 / 18)[b]

*In parenthesis is shown the number of patients who died divided by the total number of patients in the corresponding subgroup.
[a] These numbers are obtained after combining categories GSC score 3-8 and 12-9 into one category, GSC score 3-12. Thus, the number of patients treated with usual care who died is 10 + 5 = 15, and the total number of patients treated with usual care is 17 + 5 = 22.
[b] These numbers are obtained after combining categories GSC score 3-8 and 12-9 into one category, GSC score 3-12. Thus, the number of patients treated with the early resuscitation protocol who died is 4 + 10 = 14, and the total number of patients treated with the early resuscitation protocol is 7 + 11 = 18.

To calculate S-index we will use an equation-9: $S = (RR_{\text{protocol, GSC 3-12}} - 1) / [(RR_{\text{protocol, GSC ≥13}} - 1) + (RR_{\text{usual care, GSC 3-12}} - 1)]$. Furthermore, to obtain the relative risks we will divide the absolute risks per each subgroup by 22% which is the risk in patients who received usual care and had GSC score ≥13. Thus, the calculation is as follows S = (78% / 22%) / (42%/22% – 1 + 68% / 22% – 1) = 0.85 indicating a sub-additive interaction because S-index < 1.

TEMPLATE TABLE 1 Reporting the effect-measure modification analysis from multiplicative (logistic, Cox regression) models for two categorical factors.

	1st factor = 0			1st factor = 1			
	2nd factor = 0	2nd factor = 1		2nd factor = 0		2nd factor = 1	
Models adjustment	$n_{o.}/n_{pts.}$	$n_{o.}/n_{pts.}$		$n_{o.}/n_{pts.}$		$n_{o.}/n_{pts.}$	
Model 1	1 (reference)	RR(95%CI)	p-value	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 2	1 (reference)	RR(95%CI)	p-value	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 3	1 (reference)	RR(95%CI)	p-value	RR(95%CI)	p-value	RR(95%CI)	p-value

	1st factor = 1			
	2nd factor = 0		2nd factor = 1	
Models adjustment	$n_{o.}/n_{pts}$		$n_{o.}/n_{pts}$	
Model 1	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 2	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 3	RR(95%CI)	p-value	RR(95%CI)	p-value

	Effect modification: 1st factor x 2nd factor	
Models adjustment	**Additive measures**	**Multiplicative measures**
Model 1	RERI (95%CI) p-value	$RR_{1,1}$ / ($RR_{1,0}$ x $RR_{0,1}$) (95%CI) p-value
Model 2	RERI (95%CI) p-value	$RR_{1,1}$ / ($RR_{1,0}$ x $RR_{0,1}$) (95%CI) p-value
Model 3	RERI (95%CI) p-value	$RR_{1,1}$ / ($RR_{1,0}$ x $RR_{0,1}$) (95%CI) p-value

RR, risk ratio; 1st factor, the primary factor (i.e., exposure or intervention); 2nd factor, the secondary factor (i.e., exposure or intervention); RERI, relative excess risk due to interaction; 95%CI, 95% confidence interval; $n_{o.}/n_{pts.}$, number of outcomes / number of patients.
List of confounders for model 1, 2, and 3 should be noted in the footnote of the table. The RR can be replaced with odds ratio (OR) (logistic regression) or hazard ratio (HR) (Cox regression) depending on the model applied. Instead of RERI, other measures of additive effect modification can be used, such as attributable proportion (AP or modified AP), Synergy (S)-index, or their combination. The template provides example for three multivariable adjusted models, but if there are more than three models, additional rows can be added. The template provides example for 2x2 factors; if a factor has more than two subgroups additional columns can be added.*

TEMPLATE TABLE 2 Reporting the causal interaction analysis from multiplicative (logistic, Cox regression) models for two categorical factors.

	1st factor = 0			1st factor = 1		
	2nd factor = 0	2nd factor = 1		2nd factor = 0	2nd factor = 1	
Models adjustment	$n_{o.}/n_{pts.}$	$n_{o.}/n_{pts.}$		$n_{o.}/n_{pts.}$	$n_{o.}/n_{pts.}$	
Model 1	1 (reference)	RR(95%CI)	p-value	RR(95%CI) p-value	RR(95%CI)	p-value
Model 2	1 (reference)	RR(95%CI)	p-value	RR(95%CI) p-value	RR(95%CI)	p-value
Model 3	1 (reference)	RR(95%CI)	p-value	RR(95%CI) p-value	RR(95%CI)	p-value

1st factor = 1				
	2nd factor = 0		2nd factor = 1	
Models adjustment	$n_{o.}/n_{pts.}$		$n_{o.}/n_{pts.}$	
Model 1	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 2	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 3	RR(95%CI)	p-value	RR(95%CI)	p-value

2nd factor = 1				
	1st factor = 0		1st factor = 1	
Models adjustment	$n_{o.}/n_{pts.}$		$n_{o.}/n_{pts.}$	
Model 1	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 2	RR(95%CI)	p-value	RR(95%CI)	p-value
Model 3	RR(95%CI)	p-value	RR(95%CI)	p-value

Interaction: 1st factor x 2nd factor		
Models adjustment	**Additive measures**	**Multiplicative measures**
Model 1	RERI (95%CI) p-value	$RR_{1,1}$ / ($RR_{1,0}$ x $RR_{0,1}$) (95%CI) p-value
Model 2	RERI (95%CI) p-value	$RR_{1,1}$ / ($RR_{1,0}$ x $RR_{0,1}$) (95%CI) p-value
Model 2	RERI (95%CI) p-value	$RR_{1,1}$ / ($RR_{1,0}$ x $RR_{0,1}$) (95%CI) p-value

RR, risk ratio; 1st factor, the primary factor (i.e., exposure or intervention); 2nd factor, the secondary factor (i.e., exposure or intervention); RERI, relative excess risk due to interaction; 95%CI, 95% confidence interval; $n_{o.}/n_{pts.}$, number of outcomes / number of patients.

List of confounders for model 1, 2, and 3 should be noted in the footnote of the table. The RR can be replaced with odds ratio (OR) (logistic regression) or hazard ratio (HR) (Cox regression) depending on the model applied. Instead of RERI, other measures of additive effect modification can be used, such as attributable proportion (AP or modified AP), Synergy (S)-index, or their combination. The template provides example for three multivariable adjusted models, but if there are more than three models, additional rows can be added. The template provides example for 2x2 factors; if a factor has more than two subgroups additional columns can be added.*

TEMPLATE TABLE 3 Reporting the statistical interaction analysis from multiplicative (logistic, Cox regression) models if one or both factors are continuous.

Models adjustment	**1st factor**		**2nd factor**		**1st factor x 2nd factor**	
Model 1	OR(95%CI)	p-value	OR(95%CI)	p-value	OR(95%CI)	p-value
Model 2	OR(95%CI)	p-value	OR(95%CI)	p-value	OR(95%CI)	p-value
Model 3	OR(95%CI)	p-value	OR(95%CI)	p-value	OR(95%CI)	p-value

Models adjustment	**Statistical interaction: 1st factor x 2nd factor**
Model 1	RERI (95%CI) p-value
Model 2	RERI (95%CI) p-value
Model 3	RERI (95%CI) p-value

OR, odds ratio; 1st factor, the primary factor (i.e., exposure or intervention); 2nd factor, the secondary factor (i.e., exposure or intervention); RERI, relative excess risk due to interaction; 95%CI, 95% confidence interval; no. / npts., number of outcomes / number of patients.
List of confounders for model 1, 2, and 3 should be noted in the footnote of the table. The OR can be replaced with hazard ratio (HR) (Cox regression) depending on the model applied. Instead of RERI, other measures of additive effect modification can be used, such as attributable proportion (AP or modified AP), Synergy (S)-index, or their combination. The template provides example for three multivariable adjusted models, but if there are more than three models, additional rows can be added.*


SYMBIOSIS

Part II

THE ROLE OF THE KIDNEYS IN HEART FAILURE AND BEYOND

CHAPTER 4

Patient-specific Evolution of Renal function in Chronic Heart Failure Patients Dynamically Predicts Clinical Outcome in the Bio-SHiFT Study

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ABSTRACT

Background

Renal dysfunction is an important component of chronic heart failure (CHF), but its single assessment does not sufficiently reflect clinically silent progression of CHF prior to adverse clinical outcome. Therefore, we aimed to investigate temporal evolutions of glomerular and tubular markers in 263 stable CHF patients, and to determine if their patient-specific evolutions can dynamically predict clinical outcome.

Methods

We determined the risk of clinical outcome (composite endpoint of HF-hospitalization, cardiac death, LVAD-placement and heart transplantation) in relation to marker levels, slopes of their trajectories (increasing/decreasing patterns), and areas under their trajectories (AUCm). In each patient, the trajectories were estimated using repeatedly measured glomerular markers: creatinine/estimated glomerular filtration rate (eGFR), cystatin C (CysC); and tubular markers: urinary N-acetyl-beta-D-glucosaminidase (NAG) and kidney-injury-molecule (KIM)-1, plasma and urinary neutrophil-gelatinase-associated-lipocalin (NGAL).

Results

During 2.2 years of follow-up, we collected 8 (5–10) urine and 9 (5–10) plasma samples per patient. All glomerular markers predicted the endpoint (univariable hazard ratio [95% confidence interval] per 20% increase: creatinine: 1.18 [1.07–1.31], CysC: 2.41 [1.81–3.41], and per 20% eGFR decrease: 1.13 [1.05–1.23]). Tubular markers, NAG and KIM-1 also predicted the endpoint (NAG: 1.06 [1.01–1.11], and KIM-1: 1.08 [1.04–1.11]). Larger slopes were the strongest predictors (creatinine: 1.57 [1.39–1.84], eGFR: 1.59 [1.37–1.90], CysC: 1.76 [1.52–2.09]; NAG: 1.26 [1.11–1.44], and KIM-1: 1.64 [1.38–2.05]). Associations persisted after multivariable adjustment for clinical characteristics.

Conclusions

Our findings suggest that glomerular and tubular function deteriorate, but not simultaneously, during clinically silent progression of CHF. Patient-specific evolutions of these renal markers dynamically predict clinical outcome in CHF patients.

INTRODUCTION

Heart Failure (HF) is the leading cause of hospitalization worldwide.[1] Despite declines in HF-related mortality as a result of current therapies, re-hospitalization rates for decompensation of chronic heart failure (CHF) remain high.[1,2] Several blood biomarkers that predict re-hospitalization and mortality have been identified in patients with CHF.[3] Still their predictive capabilities in practice are limited, and adequate risk assessment remains a challenge.[3] Estimation of renal dysfunction, which coexists and interact with HF[3] may improve risk stratification. Baseline glomerular dysfunction, as assessed by estimated glomerular filtration rate (eGFR), entails an unfavourable prognosis in CHF.[4-6] Besides glomerular impairment, such patients often have tubular damage due to tubulo-interstitial injury by renal tissue hypoperfusion or due to damaged glomerular barrier.[7,8] Notably, a single assessment of damaged tubules predicts adverse outcome in CHF independently of eGFR.[9-11]

It is clear that both glomerular and tubular function are important in patients with CHF, but their single assessment does not sufficiently reflect deterioration along the cardio-renal axis that occurs over time preceding adverse events. Yet the temporal evolution of renal function preceding the event may dynamically ascertain the clinically silent progression of the disease. Specifically, it would enable accurate investigation of whether, and to which degree, increasing (or decreasing) levels of renal biomarkers contribute to the patient's risk, regardless of whether these levels exceed established cut-points at 'study baseline' (i.e., a random point in time prior to event).

In the context of cardio-renal interplay, patients with CHF also display large biological heterogeneity. Renal function not only changes dynamically within a patient over time, but also differs from patient to patient. Hence, the true potential of renal markers in ascertaining individual disease progression, and their accurate relation with clinical outcome, can only be revealed if their patient-specific evolutions are considered. However, detailed individual temporal evolutions of renal function in CHF have never been described.

To overcome these issues, our aim was two-fold: (1) to investigate the average (population) temporal evolutions of glomerular function (measured with plasma creatinine (Cr), eGFR and cystatin C (CysC)) and tubular status (measured with urinary kidney injury molecule (KIM)-1, N-acetyl-beta-D-glucosaminidase (NAG), and urinary and plasma neutrophil gelatinase-associated lipocalin (NGAL)) in stable patients with CHF, and (2) to determine if patient-specific (individual) evolutions of these renal biomarkers during a clinically silent period can dynamically predict clinical outcome. For this purpose we examined several aspects of the temporal evolution of each renal biomarker that may be relevant for clinical prediction.

MATERIALS AND METHODS

The *Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT)* is a prospective, observational cohort of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands. Patients were recruited during their regular visits to the Cardiology outpatient clinics of these hospitals. For this purpose, consecutive patients were screened according to the inclusion and exclusion criteria specified in Figure S1, and eligible patients were asked for informed consent. The main inclusion criteria were age ≥18 years, capability of understanding and signing informed consent, and diagnosis of CHF ≥3 months ago according to European Society of Cardiology guidelines.[12,13] Patients were ambulatory and stable, i.e., they had not been hospitalized for HF in the past three months. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients that participated in the study. This investigation comprised 263 stable patients with CHF enrolled during the first inclusion period (October 2011 until June 2013).

Baseline assessment

All patients were evaluated by research physicians, who collected information on HF-related symptoms, NYHA class, and performed a physical examination, including blood pressure, heart rate and body mass index. Information on HF etiology, left ventricular ejection fraction, cardiovascular risk factors, medical history and medical treatment was retrieved primarily from hospital records and was checked if ambiguities were present. History of cardiovascular and other comorbidities was defined as a clinical diagnosis of these conditions. Non-fasting blood and urine samples were collected, as described below.

Follow-up and study endpoints

During the study, all patients were routinely followed at the outpatient clinic by treating physicians who were blinded for biomarkers sampling and results. Study follow-up visits were predefined and scheduled every 3 months (±1 month was allowed), with a maximum of 10 study follow-up visits. At each study follow-up visit, a short medical evaluation was performed and samples were collected. All medication changes and occurrence of adverse cardiovascular events since the previous visit were recorded in electronic case report forms. During follow-up, hospitaliza-

tions for HF, MI, PCI, CABG, arrhythmias, and CVA, cardiac transplantation, left ventricular assist device (LVAD) implantation and mortality, were recorded in the electronic case report forms, and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker sampling and results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

The primary endpoint comprised the composite of cardiac death, cardiac transplantation, LVAD implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. Secondary endpoints included individual components of the primary endpoint, and also MI, PCI, CABG, CVA, and all-cause mortality. Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalization for acute or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with two of the following: BNP or NT-proBNP >3x ULN, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.[12]

Blood and urine analysis

Blood and urine samples were collected at baseline and at each study follow-up visit, and were processed and stored at a temperature of -80°C within two hours after collection. The biomarker measurements performed for this study did not lead to drug adjustments and all patients received usual care. Batch analysis of plasma and urine samples was performed at HaemoScan BV, Groningen, The Netherlands. Laboratory personnel was blinded for clinical data.

Creatinine was determined by a colorometric test by the Jaffe's reaction. Plasma was used undiluted, urine was diluted ten times in water (LLD: plasma 0,14 mg/dl, urine: 1.56 mg/ml). CysC was determined in plasma, diluted 2000 times in 0,1%BSA/PBS buffer, by ELISA (R&D systems, Minneapolis, MN) (LLD: 0.1066 µg/mL). KIM-1 was determined in urine, diluted 50% in 0,1% BSA/PBS buffer, by ELISA (R&D systems, Minneapolis, MN, USA) (LLD: 0.146 ng/mL). NAG was determined using a substrate p-nitrophenyl N-acetyl-β-D-glucosaminidase at pH 4.5 (Sigma, St Louis, MO, USA) (LLD: 0.485 U/L). NGAL was determined in urine diluted 20 times, and plasma diluted 100 times in 0,1% BSA-PBS buffer by ELISA (R&D systems, Minneapolis, MN) (LLD: urine 5.19 ng/mL, plasma 50.3 ng/mL). All urinary biomarkers were normalized to

urinary creatinine concentrations to correct for concentration or dilution of urine.

Glomerular filtration rate (GFR) was determined by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation that has been validated in HF patients.[14] Patients were categorized using National Kidney Foundation–Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines.[15]

Statistical analysis

Biomarkers measured at baseline

The association between baseline marker levels and the study endpoint was examined by Cox regression analysis. If skewed, ^{2}log-tranformation of continuous variables was used for further analyses. Analyses were first performed univariably, then statistical adjustments were performed by using two models: (1) model with biomarker of interest plus clinical variables age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, and eGFR (for tubular markers); (2) model with biomarker of interest plus biomarkers of myocardial stretch and damage, NT-proBNP and hs-cTnT. Data on all variables were complete, except for systolic blood pressure which was missing in <5% of patients and for which imputations were applied using patients' clinical and outcome data. The proportional hazards (PH) assumption was evaluated by plotting transformed Kaplan-Meier estimates, and by evaluating scaled Schoenfeld residuals.

Repeatedly measured biomarkers

We applied a joint modeling (JM) of linear mixed-effects (LME) models to assess the true underlying trajectory of a repeatedly measured marker, and a Cox survival analysis to analyze the association of this trajectory with the study endpoint. For both the fixed- and random-effects parts of LME, non-linear evolutions were tested using restricted cubic splines. If the model was not significantly improved, a linear evolution was retained. All markers were adjusted for the sampling time during follow-up. Additional statistical adjustments were as follows: (1) the repeatedly measured marker was adjusted for its baseline level (Cox model) to examine incremental value of repeated over baseline measurements (2) Cox and LME models were adjusted for the clinical variables age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, and eGFR (for tubular markers) to examine incremental value of the renal markers over the patients' clinical characteristics; (3) Cox and LME models were adjusted for biomarkers of myocardial stretch and damage (NT-proBNP and hs-cTnT) to examine the incremental value of the renal markers over these commonly used cardiac markers. Results are presented as hazard ratios (HRs) with 95%

confidence intervals (95%CI) per 20% change in biomarkers levels.

To investigate the independent predictive value of these renal markers on the study endpoints, all individual temporal biomarker patterns derived from the joint models were extracted and subsequently entered simultaneously with HF medication doses (repeatedly assessed during follow-up) into a time-dependent Cox analysis.

Parameterization of marker's trajectory

The above-described analyses estimate the instantaneous risk based on repeatedly measured marker levels. However, in the context of repeated measurements, we also estimated the following aspects:[16,17] (1) the time-dependent slope (or: rate of change) of the marker's trajectory, indicating whether and by how much the levels are increasing or decreasing at any point in time, which corresponds to the first derivative of the marker's trajectory (2) the area under the curve of the marker's trajectory (AUCm), indicating the cumulative effect of all the values the marker has taken in the past (Figure 1). The results are presented as HRs (95%CI) per 20% change in the annual slope (delta of the marker's levels/year) and the AUCm.

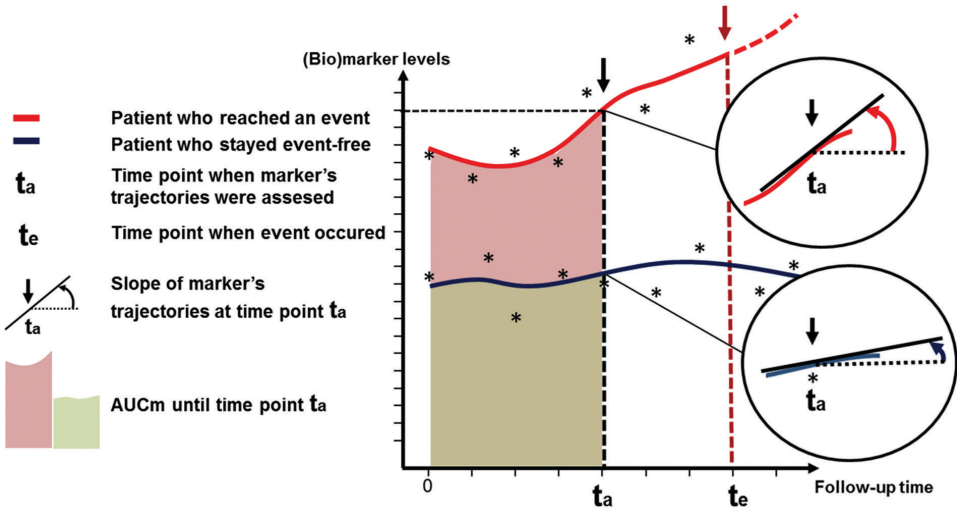

FIGURE 1 Dynamic risk prediction model using repeated marker measurements. An illustration of the underlying trajectory of a repeatedly assessed biomarker in a patient who ultimately experiences the event (solid red line) and in an event-free patient (solid blue line). Marker's levels are displayed on the y-axis and follow-up time on the x-axis. Figure shows different types of parameterization that can be examined: marker's levels at any point in time (ta), slope of the marker's trajectory at any point in time (ta), and the area under the curve of marker's trajectory (AUCm) up to the same point in time (ta). te, time when the event occurred; *, measured marker's levels.

Prospective accuracy

We determined the longitudinal marker's predictive accuracy (i.e., the ability of a marker to discriminate between a patient who experiences the event within a given time-window after the last measurement, and the patient who does not experience the event within that same time-window) using the time-dependent AUC (area under the receiver operating curve) methodology.[18] For this purpose, we chose the first year as the collection time period, and we assessed two risk time-windows: 6 and 12 months after the collection time.

All analyses were performed with R Statistical Software using package JMbayes.[17,19] All tests were two-tailed and p-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics

Table 1 displays the baseline characteristics. Patients who later experienced the endpoint, at baseline were older, more frequently had diabetes and atrial fibrillation, had lower systolic blood pressure, higher NYHA class, higher levels of NT-proBNP, cardiac troponin T, CysC, urinary NAG, and plasma NGAL, and were more frequently on diuretics than the patients who remained endpoint-free.

TABLE 1 Patient characteristics in relation to the occurrence of the composite endpoint.

Variable	Total	Composite endpoint reached		p-value
		Yes	No	
n (%)	263 (100)	70 (27)	193 (73)	
Demographics				
Age, years (mean ± SD)	67 ± 13	69 ± 13	66 ± 12	0.05
Men, n (%)	189 (72)	53 (76)	136 (70)	0.41
Clinical characteristics				
BMI, kg/m2 (mean ± SD)	27.5±4.7	27.6±4.8	27.4±4.7	0.80
Heart rate, b.p.m. (mean ± SD)	67±12	69±13	67±11	0.31
SBP, mmHg (mean ± SD)	122±20	117±17	124±21	0.02
DBP, mmHg (mean ± SD)	72±11	70±10	73±11	0.06
Features of heart failure				
NYHA class III or IV, n (%)	69 (26)	31 (44)	38 (20)	< 0.001
HF-rEF n (%)	250 (95)	66 (94)	184 (95)	0.75
HF-pEF n (%)	13 (5)	4 (6)	9 (5)	
LVEF, % (mean ± SD)	32±11	30±11	33±10	0.18

continued

Variable	Total	Composite endpoint reached		p-value
		Yes	No	
NT pro-BNP (pmol/L) †	137.3 (51.7–272.6)	282.4 (176.4–517.4)	95.3 (31.7–207.7)	< 0.001
Hs-TnT (ng/L) †	18.0 (9.5–33.2)	31.9 (20.6–49.7)	13.9 (8.4–26.7)	< 0.001
Etiology of heart failure, n (%)				
Ischemic	117 (44)	36 (51)	81 (42)	0.17
Hypertension	34 (13)	10 (14)	24 (12)	0.70
Valvular disease	12 (5)	5 (7)	7 (4)	0.23
Cardiomyopathy	68 (26)	15 (21)	53 (28)	0.32
Unknown or Others	32 (12)	4 (6)	28 (15)	
Medical history, n (%)				
Prior MI	96 (36)	32 (46)	64 (33)	0.06
Prior PCI	82 (31)	27 (39)	55 (28)	0.12
Prior CABG	43 (16)	13 (19)	30 (15)	0.57
Atrial fibrillation	106 (40)	36 (51)	70 (36)	0.03
Diabetes	81 (31)	32 (46)	49 (25)	0.002
Hypercholesterolemia	96 (36)	30 (43)	66 (34)	0.20
Hypertension	120 (46)	38 (54)	82 (42)	0.09
COPD	31 (12)	12 (17)	19 (10)	0.10
Medication use, n (%)				
Beta-blocker	236 (90)	61 (87)	175 (91)	0.40
ACE-I or ARB	245 (93)	63 (90)	182 (94)	0.22
Diuretics	237 (90)	68 (97)	169 (88)	0.02
Loop diuretics	236 (90)	68 (97)	168 (87)	0.02
Thiazides	7 (3)	3 (4)	4 (2)	0.28
Aldosterone antagonist	179 (68)	53 (76)	126 (65)	0.11
Glomerular function markers †				
Creatinine, mg/dl	1.18 (0.99–1.49)	1.30(1.02–1.52)	1.17(0.98–1.45)	0.18
eGFR, mL/min/1.73m^2	58 (43–76)	53 (40–73)	59 (44–77)	0.16
Cystatin C, mg/L	0.73 (0.57–0.97)	0.87 (0.71–1.03)	0.70 (0.53–0.90)	< 0.001
KDOQI classification, n (%)				
eGFR ≥90	28 (11)	7 (10)	21 (11)	0.18
eGFR 60-89	95 (36)	20 (28)	75 (39)	
eGFR 30-59	119 (45)	37 (53)	82 (42)	
eGFR <30	21 (8)	6 (9)	15 (8)	
Tubular markers †				
NAG, U/gCr [urine]	5.9 (3.8–9.3)	8.0 (6.0–11.0)	5.1 (3.3–8.0)	< 0.001
KIM-1, ng/gCr [urine]	477.2 (247.0–938.6)	589.0 (255.0–957.2)	465.1 (237.6–911.5)	0.10
NGAL, µg/gCr [urine]	17.4 (9.2–32.6)	18.2 (10.0–50.5)	17.4 (9.0–31.4)	0.20
NGAL, ng/ml [plasma]	190.1 (133.5–280.0)	260.8 (169.5–355.4)	179.2 (127.9–244.5)	< 0.001

BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; NYHA class, New York Heart Association class; HF-rEF, Heart failure with reduced ejection fraction; HF-pEF, heart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction; MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery

bypass grafting; CVA, cerebrovascular accident; TIA, transitory ischemic attack; COPD, chronic obstructive pulmonary disease; ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; eGFR, estimated glomerular filtration rate. Normally distributed continuous variables are presented as mean±standard deviation (SD), and non-normally distributed variables as median and interquartile range (IQR). Categorical variables are presented as numbers and percentages.
†All biomarkers levels were presented as median (IQR).

Follow-up and study endpoints

From 263 patients with CHF, a total of 1912 urine and 1984 blood samples were collected with median (IQR) of 8 (5–10) urine and 9 (5–10) plasma samples per patient. During a median (IQR) follow-up of 2.2 (1.4–2.5) years, 70 (27%) patients reached the primary endpoint: 56 patients were re-hospitalized for acute or worsened HF, 3 patients underwent heart transplantation, 2 patients underwent LVAD placement, and 9 patients died of cardiovascular causes.

Temporal evolution of glomerular function

Creatinine and eGFR

In patients who reached the composite endpoint, Cr levels on average showed an increasing pattern over time preceding the endpoint. In endpoint-free patients Cr levels were lower and remained stable during follow-up (Figure 2A). eGFR displayed similar dynamics (Figure 2B). Independently of baseline levels, repeatedly measured Cr and eGFR predicted the endpoint (per 20% increase of Cr levels: HR [95%CI] 1.18 [1.07–1.31], p=0.004, and per 20% eGFR decrease: 1.13 [1.05–1.23], p=0.002) (Table 2). Similarly, their larger slopes and larger AUCm predicted the endpoint (per 20% increase of Cr slope: 1.57 [1.39–1.84], p<0.001, per 20% decrease of eGFR slope: 1.59 [1.37–1.90], p<0.001) (per 20% increase of Cr's AUCm: 1.10 [1.03–1.18], p=0.010, and eGFR's AUCm: 1.07 [1.02–1.11], p<0.001). These risk estimates remained significant even after adjustment for clinical characteristics and dose changes of HF medications during follow-up. After adjustment for cardiac markers, Cr's levels and AUCm lost precision, whereas eGFR remained significant (Table 2). Table S1 shows similar results for HF-hospitalizations (secondary endpoint).

Cystatin C

In patients who reached the composite endpoint, CysC showed on average higher baseline levels that increased further as the endpoint approached. In endpoint -free patients, CysC levels were lower and slightly decreased during follow-up (Figure 2C).

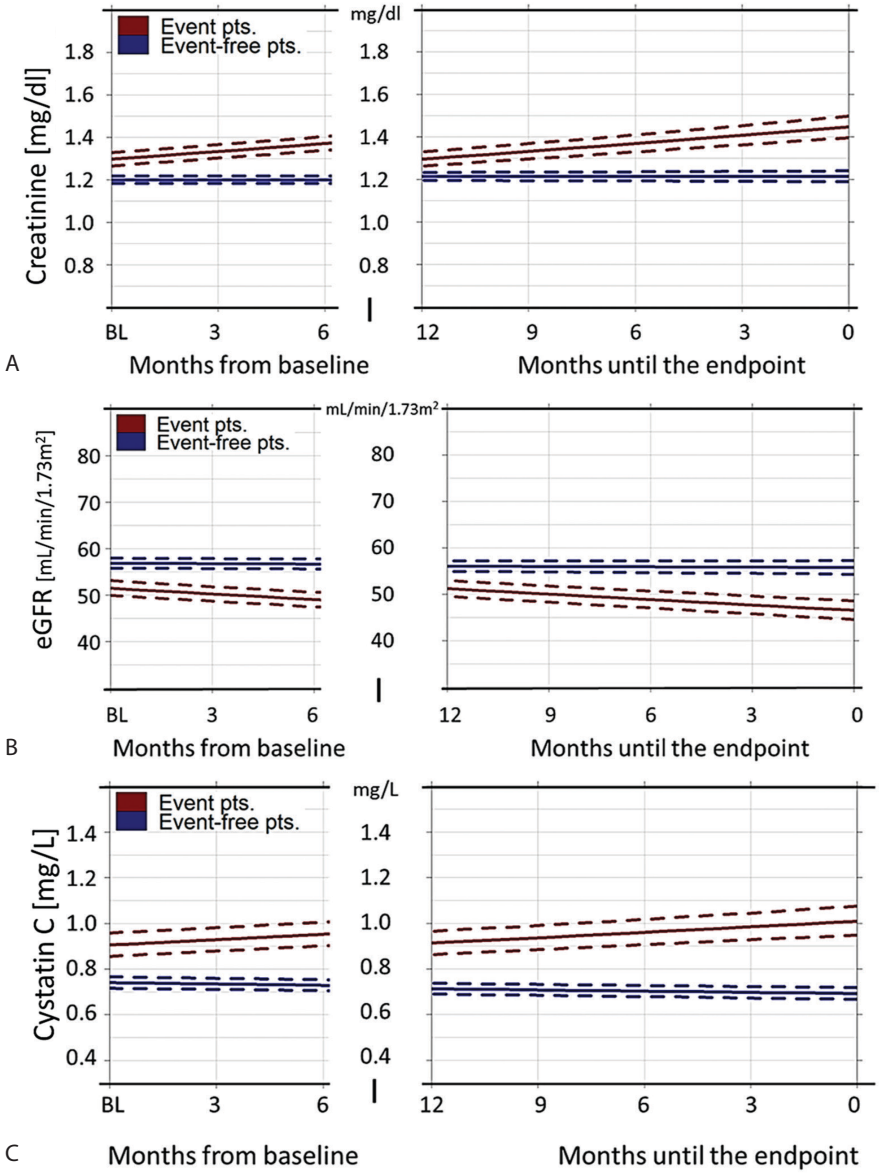

FIGURE 2 Average evolution of glomerular function markers during follow-up. Average evolution in patients who reached the study endpoint (solid red line), and in endpoint-free patients (solid blue line). Dashed lines represent the 95% confidence interval. X-axis depicts the time from baseline (left part of the x-axis), and time remaining to the event (patients who experienced incident events) or last sample moment (patients who remained event-free) (right part of the x-axis). Biomarker levels are presented on the y-axis. BL, baseline; pts., patients. **A.** creatinine (mg/dL); **B.** eGFR (ml/min/1.73m^2); **C.** cystatin C (µg/ml).

Independently of baseline levels, CysC levels at any time during follow-up were associated with the endpoint (per 20% increase of CysC levels: 2.41 [1.81–3.41], p<0.001) (Table 2). Similarly, larger slope and larger AUCm predicted the endpoint (1.76 [1.52–2.09], p<0.001 and 1.32 [1.17–1.54], p<0.001). These risk estimates remained significant after multivariable adjustments (Table 2). Table S1 shows similar results for HF-hospitalizations.

TABLE 2 Associations between glomerular function markers and the composite endpoint.

	Creatinine		eGFR		Cystatin C	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Baseline level *						
Model A	1.04 (0.99–1.09)	0.14	1.03 (0.99–1.07)	0.13	1.09 (1.05–1.14)	<0.001
Model B	1.02 (0.97–1.07)	0.49	1.02 (0.97–1.06)	0.48	1.07 (1.02–1.12)	0.007
Model C	0.98 (0.93–1.03)	0.46	0.98 (0.94–1.02)	0.28	1.00 (0.95–1.06)	0.89
Temporal evolution†						
Repeatedly measured levels						
Model 1	1.18 (1.07–1.31)	0.004	1.13 (1.05–1.23)	0.002	2.41 (1.81–3.41)	<0.001
Model 2	1.12 (1.02–1.23)	0.022	1.12 (1.06–1.20)	<0.001	2.16 (1.44–3.72)	<0.001
Model 3	1.05 (0.96–1.15)	0.28	1.09 (1.04–1.14)	<0.001	1.63 (1.35–2.30)	<0.001
Model 4	1.15 (1.08–1.24)	<0.001	1.10 (1.04–1.16)	<0.001	2.27 (1.99–2.59)	<0.001
Annual slope						
Model 1	1.57 (1.39–1.84)	<0.001	1.59 (1.37–1.90)	<0.001	1.76 (1.52–2.09)	<0.001
Model 2	1.65 (1.40–1.98)	<0.001	1.64 (1.38–2.02)	<0.001	2.00 (1.66–2.51)	<0.001
Model 3	1.37 (1.22–1.57)	<0.001	1.30 (1.16–1.46)	0.002	1.47 (1.32–1.66)	<0.001
Model 4	1.28 (1.16–1.43)	<0.001	1.18 (1.07–1.31)	0.001	1.63 (1.50–1.77)	<0.001
AUCm						
Model 1	1.10 (1.03–1.18)	0.010	1.07 (1.02–1.11)	<0.001	1.32 (1.17–1.54)	<0.001
Model 2	1.08 (1.01–1.15)	0.020	1.07 (1.02–1.12)	<0.001	1.23 (1.13–1.36)	<0.001
Model 3	1.04 (0.98–1.10)	0.17	1.06 (1.02–1.10)	<0.001	1.17 (1.08–1.28)	<0.001

AUCm – area under the curve of marker's trajectory.
* Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 20% increase of creatinine and cystatin C, and 20% eGFR decrease. **Model A**: unadjusted; **Model B**: adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, and systolic blood pressure; **Model C**: adjusted for baseline NT-proBNP and hs-cTnT.
† HRs and 95% CIs are given per 20% increase of the level, slope, and AUCm of creatinine and cystatin C, and 20% decrease of the level, slope, and AUCm of eGFR. **Model 1**: Cox model adjusted for marker's baseline levels, LME model adjusted for sampling time; **Model 2**: Cox and LME models adjusted for the clinical variables: age, sex, diabetes, atrial fibrillation,

baseline NYHA class, diuretics, systolic blood pressure, and sampling time (LME); **Model 3**: Cox and LME models adjusted for baseline NT-proBNP and hs-cTnT, and sampling time (LME); **Model 4:** Time-dependent Cox adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.

Temporal evolution of tubular function

Overall, we found substantial associations between NAG, KIM-1, and NGAL, but only mild associations between these tubular markers and glomerular function markers (namely CysC), when assessed during follow-up (Table S2).

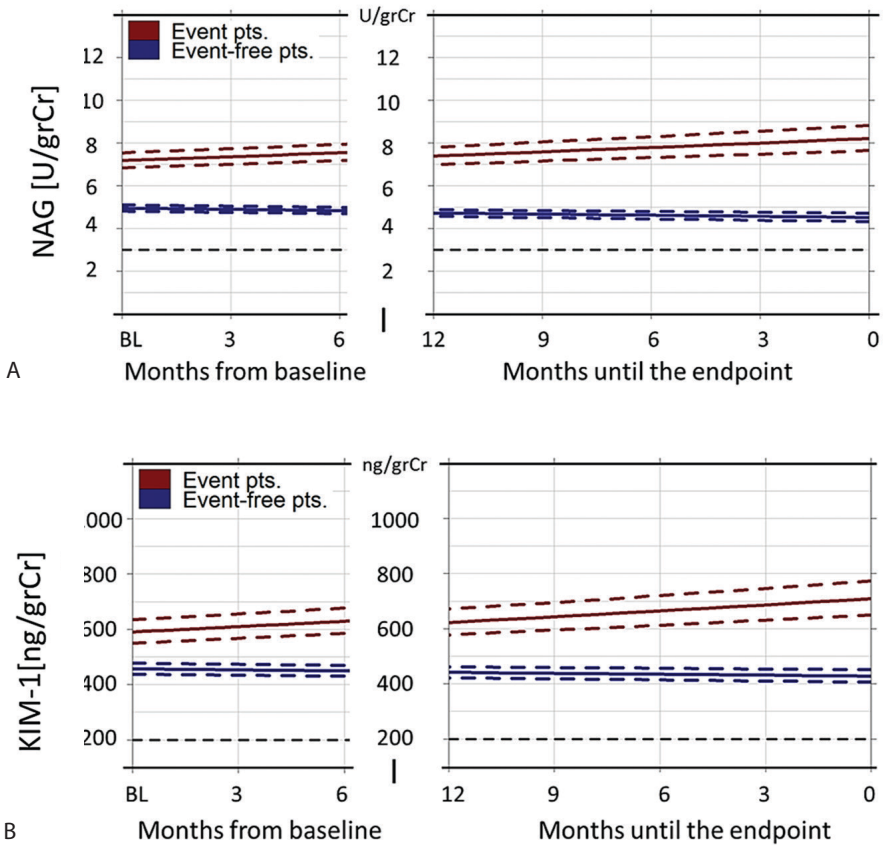

FIGURE 3 Average evolution of tubular markers, urinary NAG and KIM-1, during follow-up. For description see Figure 2. Dashed black lines represent the biomarkers' reference values. BL, baseline; pts., patients. **A.** urinary NAG (U/gCr) **B.** urinary KIM-1 (ng/gCr).

Urinary NAG

In patients who reached the composite endpoint, NAG showed on average higher baseline levels that increased further as the endpoint approached. In endpoint -free patients, NAG levels were lower and decreased during follow-up (Figure 3A). Independently of baseline levels, higher NAG levels at any time during follow-up were associated with the endpoint (per 20% increase of NAG levels: 1.06 [1.01–1.11], p=0.018). Similarly, larger NAG slope predicted the endpoint (1.26 [1.11–1.44], p=0.004).These risk estimates remained significant after multivariable adjustments, except for NAG slope that became insignificant after controlling for cardiac markers (Table 3). Table S3 shows similar results for HF-hospitalizations, except for NAG levels that lost significance after adjusting for cardiac markers.

Urinary KIM-1

In patients who reached the composite endpoint, KIM-1 levels showed an average increasing pattern over time preceding the endpoint. In endpoint-free patients, KIM-1 levels were lower and slightly decreased during follow-up (Figure 3B). Independently of baseline levels, higher KIM-1 levels at any time during follow-up were associated with the endpoint (per 20% increase of KIM-1 levels: 1.08 [1.04–1.11], p<0.001). Similarly, larger KIM-1 slope predicted the endpoint (1.64 [1.38–2.05], p<0.001). These risk estimates remained significant after multivariable adjustments (Table 3). Table S3 shows similar results for HF-hospitalizations, except for KIM-1 levels that lost significance after adjusting for cardiac markers.

Plasma and urinary NGAL

Although baseline plasma NGAL levels were higher in patients who reached the endpoint, this difference declined during follow-up (Figure S2A). The evolution of urinary NGAL levels of patients who reached the endpoint and those who did not substantially overlapped during follow-up (Figure S2B). No clear associations were found between NGAL and primary and secondary endpoints during follow-up (Tables S4 and S5).

Prospective accuracy

Table S6 shows the time-dependent AUCs for the different renal markers for the composite endpoint. After the 1-year collection time period, markers showed reasonably good discriminatory power both for the 6- and 12-month risk window

with slightly better accuracy for the 6-month window. The highest accuracy was found for clinical models using levels of CysC, NAG, and KIM-1 (6-month AUCs: 0.80, 0.81, and 0.80 respectively).

TABLE 3 Associations between tubular markers, urinary NAG and KIM-1, and the composite endpoint.

	Urinary NAG		Urinary KIM-1	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Baseline levels*				
Model A	1.07 (1.05–1.09)	<0.001	1.02 (1.00–1.04)	0.06
Model B	1.06 (1.03–1.09)	<0.001	1.01 (0.99–1.03)	0.26
Model C	1.03 (1.00–1.06)	0.050	0.99 (0.97–1.01)	0.44
Temporal evolution†				
Repeatedly measured levels				
Model 1	1.06 (1.01–1.11)	0.018	1.08 (1.04–1.11)	<0.001
Model 2	1.07 (1.03–1.12)	<0.001	1.06 (1.03–1.10)	<0.001
Model 3	1.05 (1.00–1.10)	0.048	1.04 (1.01–1.07)	0.016
Model 4	1.13 (1.09–1.17)	<0.001	1.06 (1.03–1.09)	<0.001
Annual slope				
Model 1	1.26 (1.11–1.44)	0.004	1.64 (1.38–2.05)	<0.001
Model 2	1.50 (1.18–2.00)	0.002	1.78 (1.41–2.39)	<0.001
Model 3	0.81 (0.65–1.41)	0.16	1.52 (1.25–1.98)	<0.001
Model 4	1.10 (1.02–1.20)	0.009	1.12 (1.04–1.20)	0.002
AUCm				
Model 1	1.02 (0.99–1.05)	0.11	1.01(0.99–1.02)	0.23
Model 2	1.04 (1.01–1.07)	0.01	1.01 (0.99–1.03)	0.10
Model 3	1.01 (0.98–1.05)	0.33	1.01 (0.99–1.02)	0.38

AUCm – area under the curve of marker's trajectory.
* Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 20% increase of urinary NAG and KIM-1. **Model A**: unadjusted; **Model B**: adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and eGFR; **Model C**: adjusted for baseline NT-proBNP and hs-cTnT.
† HRs and 95% CIs are given per 20% increase of the level, slope, and AUCm of urinary NAG and KIM-1. **Model 1**: Cox model adjusted for marker's baseline levels, LME model adjusted for sampling time; **Model 2**: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, eGFR, and sampling time (LME); **Model 3**: Cox and LME models adjusted for baseline NT-proBNP and hs-cTnT, and sampling time (LME). **Model 4**: Time-dependent Cox adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.

Patient-specific dynamic prediction

Figure S3 shows the temporal patterns of eGFR and NAG in several individual patients from our cohort, together with their corresponding individual survival probabilities as estimated by the joint model. The figure shows that each time an additional measurement is performed in the patient, the individual survival probability is updated. Specifically, rising marker levels and worsening prognosis can be seen in the example patients who ultimately reached the composite endpoint, versus stable or decreasing marker levels and more favorable prognosis in the example patients who stayed event-free.

DISCUSSION

We have shown that in patients with CHF both glomerular function (as assessed by repeatedly measured creatinine, eGFR, and CysC), and tubular function (as assessed by repeatedly measured urinary NAG and KIM-1) deteriorate over time preceding clinical outcome. Importantly, patient-specific trajectories of all glomerular markers dynamically predicted the event, and CysC was the strongest predictor. Similarly, patient-specific trajectories of urinary NAG and KIM-1 indicated progression of tubular damage in patients who later suffered adverse events. No clear associations were found between repeatedly measured plasma or urinary NGAL and the event. Therefore, the current study does not justify its use for clinical prediction in patients with CHF.

Our findings confirm that renal function is an indivisible component of HF, and that it is clinically relevant for the monitoring of stable patients with CHF. Importantly, our results show that temporal changes in renal function remain predictive for clinical outcome despite controlling for NYHA class, cardiac markers and other clinical features, which suggests that renal dysfunction may drive adverse clinical outcomes independently of cardiac dysfunction. In addition, the results demonstrate the predictive value not only of GFR levels (single value or cumulative effects), but also of GFR slope. These findings are supported by other studies.[4,10] However, unlike previous studies, our study underscores that GFR evolution should be assessed as a function of time. In other words, information on early and late GFR changes,[20] as well as the time interval during which GFR was measured should be taken into consideration. This recommendation is also supported by recent results from Damman et al, who found that when eGFR is assessed as a function of time, any decrease in eGFR will result in increased event rates. In previous studies, deltas in creatinine or eGFR between any two sampling moments were mostly used, which may have led to bias as a consequence of differences in the time-periods (before the event) in which sampling was performed. In our study, the ob-

servations were made using two glomerular markers, creatinine and CysC, which were assessed at fixed time intervals; using more than twice as many repeated measurements as previous studies did. Notably, CysC showed the strongest association with adverse events. Considering that generation of creatinine changes when muscle wasting occurs with progression of cardiac disease, this can be of particular interest when renal function is repeatedly assessed in the same individual with CHF. Nonetheless, this issue requires further exploration.

In the setting of tubular injury, we found not only that patients with CHF experience tubular damage, but also that the damage progresses over time (months) preceding a clinical event. This extends previous findings by demonstrating that tubular markers, which were previously shown to capture acute kidney injury[21], are also clinically relevant in chronic tubular damage in patients with CHF when followed during a prolonged time period.[11] To our best knowledge, our study is the first to simultaneously follow glomerular and tubular markers and to show that glomerular dysfunction and tubular injury, in most cases, do not progress over time in parallel. This implies that, although the failing heart affects both renal compartments, the degree of damage in these compartments is usually not temporally coupled. Therefore, they should be viewed as different renal entities in CHF. In addition, when we examined NAG and KIM-1, we found that NAG levels will rise first, followed by a rise in KIM-1. This suggests that, although both markers are labeled as "tubular damage markers", they reflect different biological aspects of tubular injury, and their values depend on the moment in time prior to the event at which they are assessed. These findings are in line with their behavior as previously found. Increased urinary excretion of NAG has been found to occur with abnormal increases in protein traffic across the proximal tubules as a consequence of a damaged glomerular barrier.[22] On the other hand, KIM-1 gene expression has been found to be up-regulated in a dose-dependent manner in response to direct tubular injury.[23] KIM-1 also correlated strongest with tubular damage as determined by kidney biopsies. It outperformed serum creatinine, blood urea nitrogen (BUN) and urinary NAG.[24,25] Thus, it appears that NAG is a marker of tubular dysfunction that shows an early initial rise, while KIM-1 can serve as a quantitative marker of tubular damage, if modeled in a time-dependent manner. Importantly, both tubular markers are relevant for clinical outcomes.

The unique advantages of our study include frequent repeated measurements at pre-specified time intervals (i.e., sampling was not left at the discretion of the treating physicians) during longer-term follow-up. This allowed us to provide an unbiased assessment of a patient's risk by using the complete temporal biomarker trajectory as assessed over the entire follow-up period. Based on this underlying trajectory,

biomarker levels are used to estimate the risk of future adverse events.[19] Herewith, a window of opportunity may be gained to modify the treatment before a future event occurs. Joint modeling (JM) of patient-specific marker trajectories and survival analysis enables us to perform individualized risk predictions based on individual biomarker values. Subsequently, predictions are dynamically updated to provide real-time risk assessment whenever extra information is collected.[18] Such dynamic risk profiling can enable physicians to better detect disease progression and to make well-informed individualized treatment decisions. Applicability of JM in daily practice is user-friendly, and an app is already available into which a patient's data (baseline and follow-up) can be uploaded (for details please see Figure S4).[26]

Study limitations

Firstly, our cohort consisted mainly of HFrEF patients. The low number of patients with HFpEF can most likely be attributed to the fact that in the Netherlands, most HFpEF patients are treated by the general practitioner or in secondary referral centres, while the current study was performed in two centres which were both tertiary referral centres. Potential inclusion bias is not a likely reason for the low HpEF rate, because all consecutive patients were screened in both participating centres. Secondly, enrolled CHF patients were in a better health condition than previously reported CHF populations. Yet we were able to demonstrate, even in this 'less sick' CHF population, that evolutions of glomerular and tubular dysfunction predict clinical outcome. Thus, it is possible that these markers could perform even better in more sick CHF patients. Thirdly, although we adjusted for several confounders, residual confounding may be present. However, we corrected all urinary markers for concentration or dilution of urine caused by diuretics during follow-up. Furthermore, treating physicians were blinded to biomarker data to exclude bias by treatment effect. Finally, although our findings underscore the importance of regular monitoring of both glomerular and tubular function in CHF, routine evaluation of kidneys should always be seen in the light of the patient's clinical status.

CONCLUSION

Altogether, our findings demonstrate that glomerular function (as assessed by creatinine, eGFR, and CysC), and tubular function (as assessed by urinary NAG and KIM-1) deteriorate, but not simultaneously, during clinically silent progression of CHF over time preceding adverse events. Patient-specific temporal evolutions of these repeatedly measured renal markers dynamically predict clinical outcome in CHF patients, and are useful for individual risk profiling.

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SUPPLEMETARY INFORMATION

Inclusion criteria

1. Age ≥ 18 years?
2. Diagnosis Chronic Heart Failure ≥ 3 months*?
3. Written informed consent?

***Heart Failure, diagnosed ≥ 3 months ago according to the definition of the European Society of Cardiology (ESC), which includes the following features:**

- Symptoms typical of heart failure: breathlessness at rest or on exercise, fatigue, tiredness, ankle swelling
  AND
- Signs typical of heart failure: tachycardia, tachypnea, pulmonary rales, pleural effusion, raised jugular venous pressure, peripheral edema, hepatomegaly
  AND
- Objective evidence of a structural or functional abnormality of the heart at rest: cardiomegaly, third heart sound, cardiac murmurs, abnormality on the echocardiogram, raised natriuretic peptide concentration.

Heart Failure with preserved ejection fraction (HFpEF) diagnosed ≥ 3 months ago according to the definition of the ESC, which includes the following features:

- Presence of signs and/or symptoms of heart failure (as described above)
  AND
- Presence of normal or only mildly abnormal ejection fraction (LVEF ≥50%)
  AND
- Evidence of diastolic left ventricular dysfunction according to the criteria of the ESC:
  - E/E' >15
    OR
  - 15> E/E' >8 AND NT-proBNP >220 pg/ml / BNP >200 pg/ml
    OR
  - E/A <0.5 and DT >260 mm
    OR
  - Atrial fibrillation

Exclusion criteria

1. Heart Failure secondary to circulatory high-output conditions
2. Scheduled for surgery or intervention for both coronary and non-coronary indications within 6 months from inclusion
3. Severe renal failure for which dialysis is needed
4. Known moderate or severe liver disease
5. COPD Gold stage IV
6. Coexistent condition with life expectancy ≤ 1 year
7. Congenital heart disease

FIGURE S1 Inclusion and exclusion criteria.

TABLE S1 Associations between glomerular function markers and HF-hospitalizations.

	Creatinine		eGFR		Cystatin C	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Baseline level *						
Model A	1.04 (0.98–1.10)	0.17	1.04 (0.99–1.08)	0.11	1.10 (1.05–1.15)	<0.001
Model B	1.01 (0.95–1.08)	0.70	1.01 (0.96–1.07)	0.65	1.06 (1.01–1.12)	0.034
Model C	0.98 (0.92–1.04)	0.46	0.98 (0.93–1.03)	0.38	1.01 (0.95–1.07)	0.85
Temporal evolution†						
Repeatedly measured levels						
Model 1	1.22 (1.09–1.39)	<0.001	1.17 (1.08–1.27)	<0.001	2.40 (1.79–3.26)	<0.001
Model 2	1.13 (1.01–1.27)	0.032	1.15 (1.07–1.24)	<0.001	2.64 (1.63–4.31)	<0.001
Model 3	1.07 (0.96–1.18)	0.21	1.12 (1.06–1.18)	<0.001	2.04 (1.46–3.31)	<0.001
Model 4	1.19 (1.10–1.28)	<0.001	1.12 (1.06–1.19)	<0.001	2.96 (2.46–3.56)	<0.001
Annual slope						
Model 1	1.61 (1.42–1.86)	<0.001	1.65 (1.41–2.00)	<0.001	1.75 (1.50–2.05)	<0.001
Model 2	1.76 (1.45–2.17)	<0.001	1.68 (1.42–2.12)	<0.001	1.93 (1.61–2.3)	<0.001
Model 3	1.43 (1.27–1.62)	<0.001	1.36 (1.21–1.55)	<0.001	1.46 (1.31–1.68)	<0.001
Model 4	1.36 (1.21–1.52)	<0.001	1.27 (1.14–1.41)	<0.001	1.65 (1.51–1.81)	<0.001
AUCm						
Model 1	1.10 (1.02–1.19)	0.014	1.07 (1.02–1.12)	0.004	1.35 (1.7–1.63)	<0.001
Model 2	1.08 (1.01–1.16)	0.026	1.08 (1.03–1.12)	0.004	1.22 (1.11–1.38)	<0.001
Model 3	1.05 (0.98–1.12)	0.18	1.07 (1.03–1.11)	<0.01	1.20 (1.09–1.33)	<0.001

AUCm – area under the curve of marker's trajectory.
* Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 20% increase of creatinine and cystatin C, and 20% eGFR decrease. **Model A**: unadjusted; **Model B**: adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, and systolic blood pressure; **Model C**: adjusted for baseline NT-proBNP and hs-cTnT.
† HRs and 95% CIs are given per 20% increase of the level, slope, and AUCm of creatinine and cystatin C, and 20% decrease of the level, slope, and AUCm of eGFR. **Model 1**: Cox model adjusted for marker's baseline levels, LME model adjusted for sampling time; **Model 2**: Cox and LME models adjusted for the clinical variables: age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and sampling time (LME); **Model 3**: Cox and LME models adjusted for NT-proBNP and hs-cTnT, and sampling time (LME); **Model 4**: Time-dependent Cox adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.

TABLE S2 **Associations between glomerular and tubular renal markers.**

	Dependent variable* – Tubular markers (%)							
	KIM-1		NAG		NGAL urine		NGAL plasma	
Independent variable*	**ß (95%CI)**	**p-value**	**ß (95%CI)**	**p-value**	**ß (95%CI)**	**p-value**	**ß (95%CI)**	**p-value**
Tubular markers								
KIM-1			60 (50 to 68)	<0.001	44 (34 to 54)	<0.001	8 (4 to 12)	<0.001
NAG	52 (44 to 60)	<0.001			52 (42 to 62)	<0.001	8 (4 to 12)	<0.001
NGAL urine	32 (24 to 38)	<0.001	42 (36 to 50)	<0.001			6 (2 to 10)	0.004
NGAL plasma	46 (28 to 64)	<0.001	68 (50 to 86)	<0.001	46 (24 to 68)	<0.001		
Glomerular function markers								
Creatinine	-2 (-2 to 19)	0.79	22 (0 to 44)	0.05	16 (-8 to 40)	0.21	52 (42 to 62)	<0.001
Cystatin C	24 (6 to 42)	0.012	24 (6 to 44)	0.01	56 (36 to 78)	<0.001	70 (62 to 78)	<0.001
eGFR	2 (-16 to 20)	0.83	-26 (-44 to -6)	0.01	-30 (-52 to -8)	0.005	-46 (-54 to -16)	<0.001

* Beta coefficients and corresponding 95% confidence interval (CI) were calculated using linear mixed-effects models and are presented as percentage (%) change of dependent variable per doubling of independent variable at the same time point during follow-up.

TABLE S3 Associations between tubular damage markers, urinary NAG and KIM-1, and HF-hospitalizations.

	Urinary NAG		Urinary KIM-1	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Baseline levels*				
Model A	1.07 (1.05–1.10)	<0.001	1.01 (0.99–1.04)	0.16
Model B	1.07 (1.04–1.10)	<0.001	1.01 (0.98–1.03)	0.55
Model C	1.04 (1.01–1.07)	0.020	0.99 (0.95–1.01)	0.26
Temporal evolution†				
Repeatedly measured levels				
Model 1	1.09 (1.02–1.11)	0.006	1.06 (1.02–1.10)	0.006
Model 2	1.08 (1.03–1.12)	0.002	1.04 (1.01–1.08)	0.006
Model 3	1.03 (0.98–1.07)	0.29	1.03 (0.99–1.06)	0.09
Model 4	1.13 (1.09–1.17)	<0.001	1.07 (1.04–1.10)	<0.001
Annual slope				
Model 1	1.48 (1.21–1.99)	<0.001	1.65 (1.35–2.10)	<0.001
Model 2	1.80 (1.33–2.69)	<0.001	1.71 (1.35–2.25)	<0.001
Model 3	0.93 (0.80–1.18)	0.40	1.25 (1.13–1.39)	<0.001
Model 4	1.08 (1.00–1.18)	0.06	1.16 (1.08–1.25)	<0.001
AUCm				
Model 1	1.03 (0.99–1.06)	0.09	1.00 (0.99–1.02)	0.59
Model 2	1.04 (1.01–1.08)	0.020	1.01 (0.99–1.03)	0.31
Model 3	1.00 (0.97–1.03)	0.98	1.00 (0.98–1.02)	0.74

AUCm – area under the curve of marker's trajectory.
* Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 20% increase in urinary NAG and KIM-1. **Model A**: unadjusted; **Model B**: adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and eGFR; **Model C**: adjusted for baseline NT-proBNP and hs-cTnT.
† HRs and 95% CIs are given per 20% increase in the level, slope, and AUCm of urinary NAG and KIM-1. **Model 1**: Cox model adjusted for marker's baseline levels; **Model 2**: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, eGFR, and sampling time (LME); **Model 3**: Cox and LME models adjusted for baseline NT-proBNP and hs-cTnT, and sampling time (LME); **Model 4**: Time-dependent Cox adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.

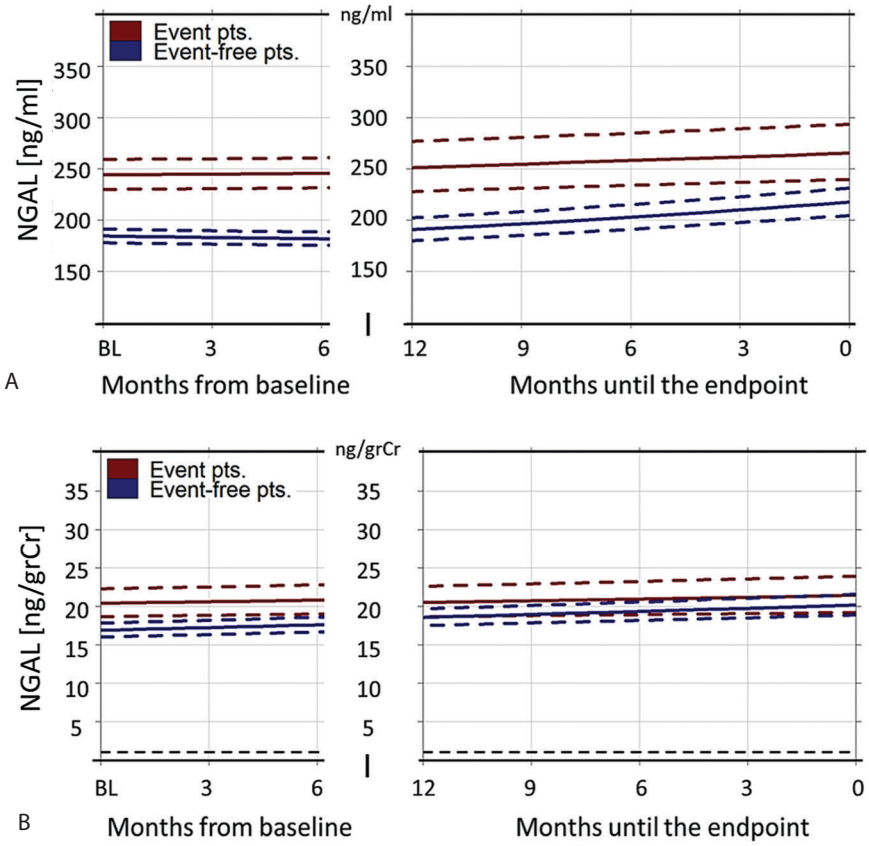


FIGURE S2 Average evolution of tubular markers, urinary and plasma NGAL, during follow-up. Average evolution in patients who reached the study endpoint (solid red line), and in event-free patients (solid blue line). Dashed lines represent the 95% confidence interval. X-axis depicts the time from baseline (left part of the x-axis), and the time remaining to the event (patients who experienced incident events) or last sampling moment (patients who remained event-free) (right part of the x-axis). Biomarker levels are presented on the y-axis. Dashed black lines represent the biomarkers' reference values (<1 µg/gCr). **A.** plasma NGAL(ng/ml); **B.** urinary NGAL(µg/gCr). BL, baseline; pts., patients.

TABLE S4 Associations between tubular markers, urinary and plasma NGAL, and the composite endpoint.

	urinary NGAL		plasma NGAL	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Baseline levels*				
Model A	1.01 (1.00–1.02)	0.08	1.08 (1.04–1.11)	<0.001
Model B	1.01 (0.99–1.03)	0.21	1.06 (1.02–1.10)	0.004
Model C	0.99 (0.97–1.01)	0.95	1.01 (0.97–1.04)	0.74
Temporal evolution†				
Repeatedly measured levels				
Model 1	0.94 (0.65–1.35)	0.78	1.01 (0.94–1.09)	0.75
Model 2	X		x	
Model 3	x		x	
Annual slope				
Model 1	x		x	
Model 2	x		x	
Model 3	x		x	
Model 4	x		x	
AUCm				
Model 1	x		x	
Model 2	x		x	
Model 3	x		x	

AUCm – area under the curve of marker's trajectory.
* Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 20% increase of urinary and plasma NGAL. **Model A**: unadjusted; **Model B**: adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and eGFR; **Model C**: adjusted for baseline NT-proBNP and hs-cTnT.
† HRs and 95% CIs are given per 20% increase of the level, slope, and AUCm of urinary and plasma NGAL. **Model 1**: Cox model adjusted for marker's baseline levels, LME model adjusted for sampling time; **Model 2**: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, eGFR, and sampling time (LME); **Model 3**: Cox and LME models adjusted for baseline NT-proBNP and hs-cTnT, and sampling time (LME). **Model 4**: Time-dependent Cox adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.
x The models were not performed because repeatedly measured level was not significant.

TABLE S5 Associations between tubular markers, urinary and plasma NGAL, and HF-hospitalizations.

	urinary NGAL		plasma NGAL	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Baseline levels*				
Model A	1.02 (1.00–1.03)	0.06	1.09 (1.05–1.13)	<0.001
Model B	1.01 (0.99–1.03)	0.26	1.06 (1.02–1.10)	0.007
Model C	1.00 (0.99–1.02)	0.90	1.01 (0.97–1.06)	0.49
Temporal evolution†				
Repeatedly measured levels				
Model 1	0.99 (0.95–1.03)	0.68	0.99 (0.91–1.09)	0.84
Model 2	x		x	
Model 3	x		x	
Model 4	x		x	
Annual slope				
Model 1	x		x	
Model 2	x		x	
Model 3	x		x	
Model 4	x		x	
AUCm				
Model 1	x		x	
Model 2	x		x	
Model 3	X		x	

AUCm – area under the curve of marker's trajectory.
* Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 20% increase of urinary and plasma NGAL. **Model A**: unadjusted; **Model B**: adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and eGFR; **Model C**: adjusted for baseline NT-proBNP and hs-cTnT.
† HRs and 95% CIs are given per 20% increase of the level, slope, and AUCm of urinary and plasma NGAL. **Model 1**: Cox model adjusted for marker's baseline levels, LME model adjusted for sampling time; **Model 2**: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, eGFR, and sampling time (LME); **Model 3**: Cox and LME models adjusted for baseline NT-proBNP and hs-cTnT, and sampling time (LME). **Model 4**: Time-dependent Cox adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.
x The models were not performed because repeatedly measured level was not significant.

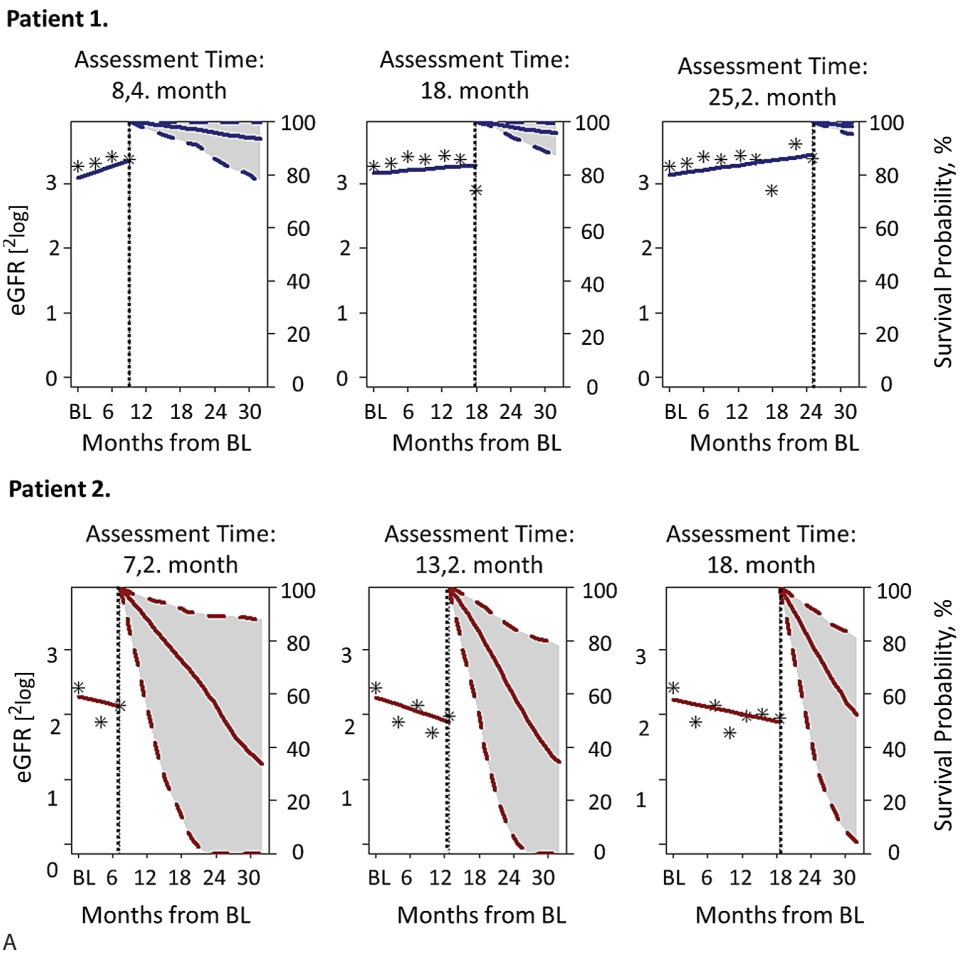
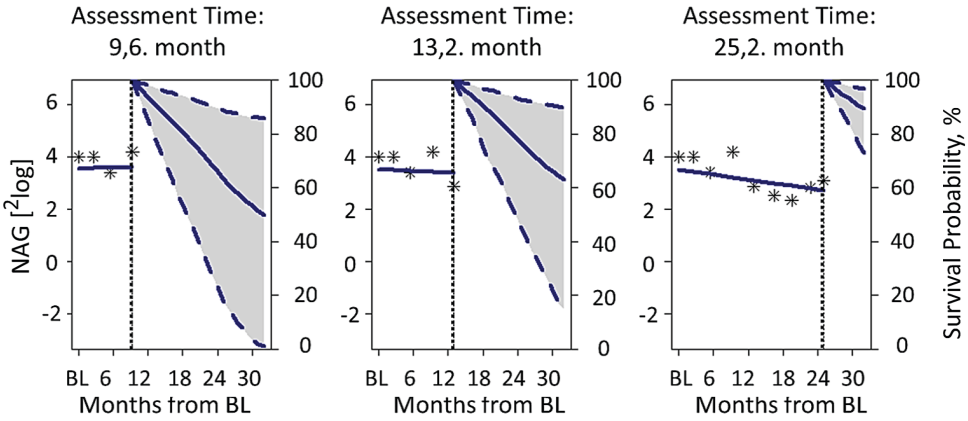
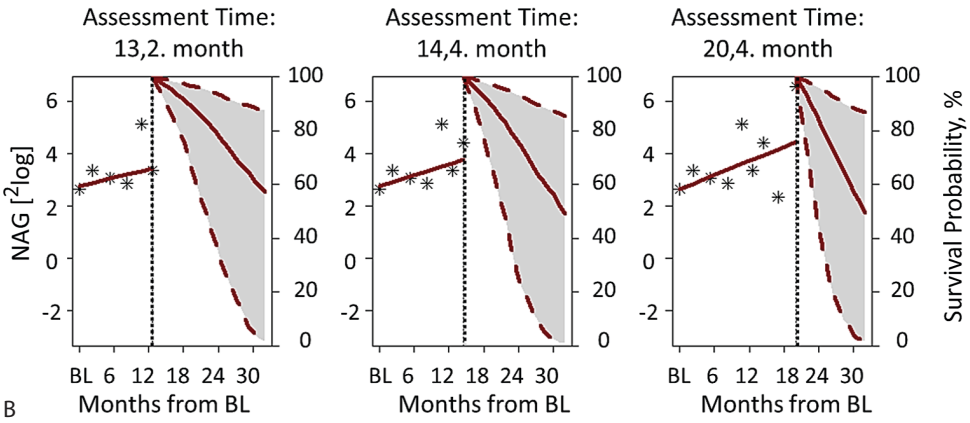


FIGURE S3 Clinical scenarios where a patient's risk is dynamically profiled using patient-specific trajectories. The solid red lines depict patients who experienced the study endpoint, and the solid blue lines depict patients who did not. X-axis depicts follow-up time in months starting from baseline (BL). Biomarker levels (on ²log scale) are displayed on the primary (left) Y-axis and survival probability (%) on the secondary (right) Y-axis. *Patient-specific marker's trajectory* with scatter points is displayed left of the vertical dotted black line. To the right of this line, the corresponding *conditional survival probability curve* is displayed with 95% confidence intervals (grey area). To show how this conditional survival probability curve is dynamically updated every time an extra measurement is recorded, we have provided three time-points at which the risk was assessed. For each of the four patients, we considered: (1) information on their measurements up to these three time-points and (2) the fact that they had survived up to each of the time-points. This information was then jointly modeled to provide the conditional survival probability curve for the remaining time period until the study ended (i.e., the patients suffered the event or were censored).
*Conditional – given that the patient survived up to the time interval during which measurements were collected.

Patient 3.



Patient 4.



B

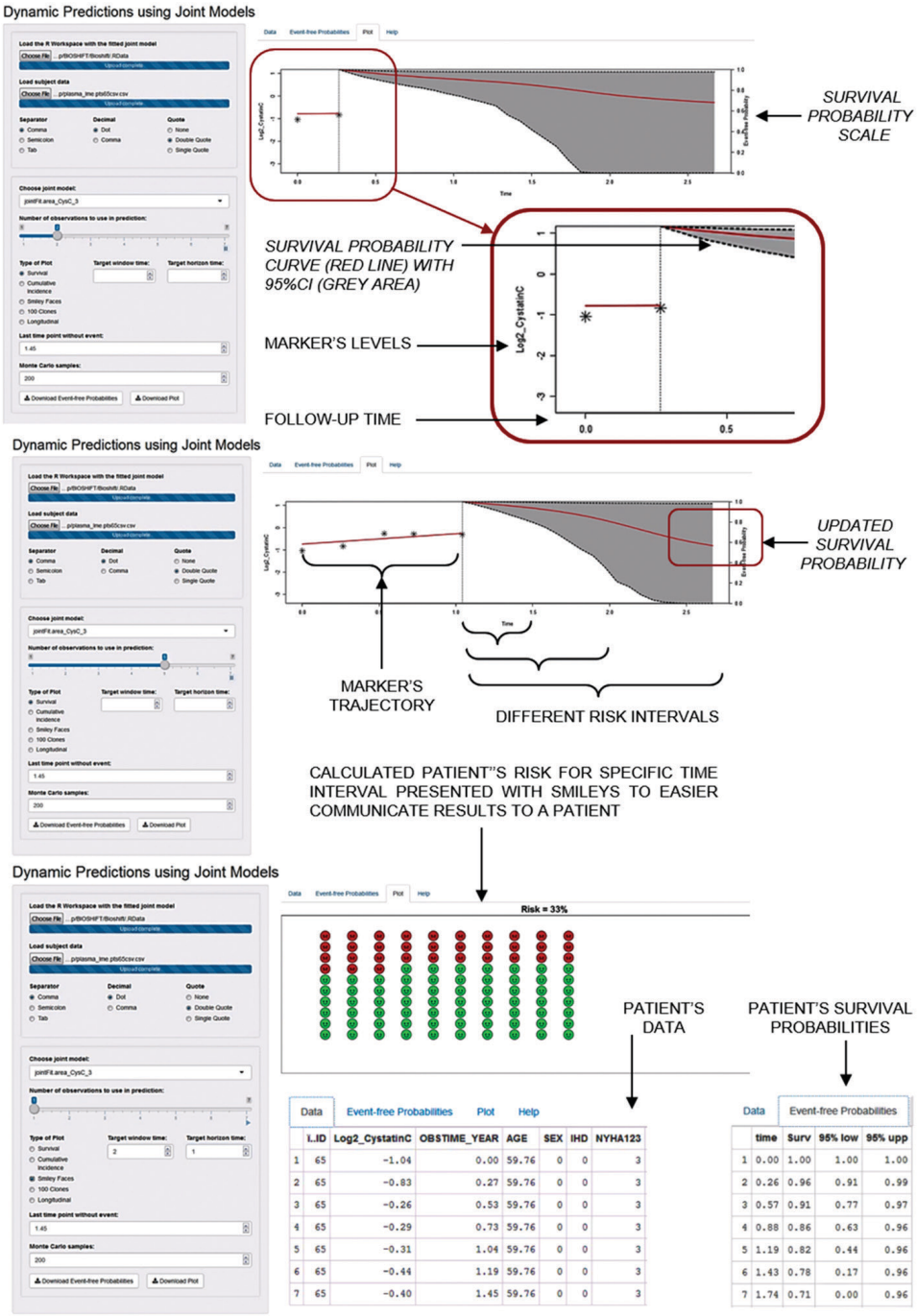
Scenario A. For the first patient (who did not experience the endpoint), we notice high baseline eGFR levels and high conditional survival probability. Conversely, the second patient (who ultimately experienced the endpoint) exhibits lower baseline eGFR levels, that continue to decline during follow-up. This eGFR decline corresponds to decline in the patient's conditional survival probability.

Scenario B. For the third patient (who did not experience the endpoint), we notice slightly higher NAG levels than for the fourth patient (who ultimately experienced the endpoint) at the moment of the first assessment. Logically, the conditional survival probability for the third patient is slightly lower than for the fourth patient. Yet the third patient exhibits a decline in NAG levels during follow-up, and the patient's conditional survival probability profile improves. Conversely, in the fourth patient NAG levels increase over time preceding the endpoint, which reduces the patient's survival probability.

TABLE S6 The longitudinal marker's accuracy.

Renal Markers	Risk Time Window	AUC (t)	
		Clinical model	Biomarkers model
Repeatedly measured levels			
Creatinine			
	6 months	0.77	0.75
	12 months	0.72	0.76
eGFR			
	6 months	0.77	0.70
	12 months	0.73	0.72
Cystatin C			
	6 months	0.80	0.77
	12 months	0.74	0.72
NAG			
	6 months	0.81	0.77
	12 months	0.76	0.79
KIM-1			
	6 months	0.80	0.75
	12 months	0.72	0.76
Annual slope			
Creatinine			
	6 months	0.64	0.62
	12 months	0.67	0.69
eGFR			
	6 months	0.64	0.62
	12 months	0.68	0.69
Cystatin C			
	6 months	0.78	0.77
	12 months	0.71	0.72
NAG			
	6 months	0.76	0.73
	12 months	0.73	0.71
KIM-1			
	6 months	0.61	0.66
	12 months	0.65	0.72

We determined the longitudinal marker's predictive accuracy (i.e., an ability of a marker to discriminate between a patient who experiences the endpoint within a given risk time window after the last measurement, and the patient who does not experience the event within the same risk time window) using the time-dependent AUC. For this purpose, we chose the first year as the collection time period, and we assessed two risk time windows: 6 and 12 months after collection time. We determined the predictive accuracy of the marker's levels and slopes in two multivariable adjusted models: a) clinical model: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, eGFR (for NAG and KIM-1), and sampling time (LME); b) biomarker model: Cox and LME models adjusted for NT-proBNP and hs-cTnT, and sampling time (LME).



	I_ID	Log2_CystatinC	OBSTIME_YEAR	AGE	SEX	IHD	NYHA123
1	65	-1.04	0.00	59.76	0	0	3
2	65	-0.83	0.27	59.76	0	0	3
3	65	-0.26	0.53	59.76	0	0	3
4	65	-0.29	0.73	59.76	0	0	3
5	65	-0.31	1.04	59.76	0	0	3
6	65	-0.44	1.19	59.76	0	0	3
7	65	-0.40	1.45	59.76	0	0	3

	time	Surv	95% low	95% upp
1	0.00	1.00	1.00	1.00
2	0.26	0.96	0.91	0.99
3	0.57	0.91	0.77	0.97
4	0.88	0.86	0.63	0.96
5	1.19	0.82	0.44	0.96
6	1.43	0.78	0.17	0.96
7	1.74	0.71	0.00	0.96

FIGURE S4 An app interface using joint modeling approach to calculate and communicate the risk in an individual patient.

CHAPTER 5

Glomerular Decline and Progressive Tubular Damage in Chronic Heart Failure: Clinical Determinants and Combined Value for Prognosis The Bio-SHiFT Study

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Submitted

ABSTRACT

Background

Progressive tubular damage (PTD) and glomerular decline (GD) affect prognosis in chronic heart failure (CHF). We investigated clinical determinants of PTD and GD and their combined prognostic value for CHF patients.

Methods

In 263 patients, during 2.2-years, we prospectively collected 9-blood and 8-urine samples per patient. We determined slopes (biomarker change/year) of urinary tubular damage markers (N-acetyl-beta-D-glucosaminidase [uNAG], kidney injury molecule [uKIM]-1) and plasma creatinine (Cr). PTD was categorized according to uNAG or uKIM-1 (increase in neither, increase in either, and increase in both). GD was defined as increasing Cr slope. The endpoint comprised HF-hospitalization, cardiac death, LVAD-placement, and heart transplantation.

Results

Higher baseline NT-proBNP and lower eGFR independently predicted PTD (per doubling NT-proBNP: OR 1.26 [95%CI:1.07-1.49]; per 10mL/min/1.73m^2 eGFR decrease 1.16 [1.03-1.31]). Higher loop diuretic doses, lower MRA doses, and higher eGFR independently predicted GD (furosemide: per 40mg increase: 1.32 [1.08-1.62]; spironolactone: per 25mg decrease: 1.76 [1.07-2.89]; eGFR: per 10mL/min/1.73m^2 increase: 1.40 [1.20-1.63]). Lack of PTD inferred highest survival regardless of GD, but PTD and GD combined entailed poorest survival.

Conclusions

PTD and GD are associated with different clinical determinants of CHF patients. They carry the poorest prognosis when they deteriorate concurrently. PTD may be prognostically important even when glomerular function appears intact.

INTRODUCTION

Renal dysfunction is the most prevalent comorbidity among patients with chronic heart failure (CHF), and is strongly associated with clinical outcomes such as HF-related hospitalization and mortality.[1-3] Underlying hemodynamic dependence between the heart and the kidneys is widely considered as the main driver of the cardiorenal interaction leading to adverse outcomes.[4] However, other biochemical, neurohumoral, and immunological derangements also occur during the organs' interplay, which has led to the definition of cardiorenal syndrome (CRS).[5]

Because renal dysfunction entails poor prognosis in CHF, attention has focused on identifying the signals along the cardio-renal axis that precede adverse outcomes.[6] Yet, the mechanisms and the chronology according to which the failing heart damages specific renal structures that lead to CRS are poorly understood.[7] Decreased baseline glomerular function is clearly important, but glomerular decline (GD) quantified as creatinine increase over time has been shown to be an even more prominent predictor.[1] We have recently confirmed and extended these findings by using frequent, repeated GD assessment in CHF patients.[8]

Besides glomerular dysfunction, tubular damage is often present in CHF due to tubulo-interstitial injury by renal tissue hypoperfusion or due to a damaged glomerular filtration barrier.[9-11] Higher levels of tubular damage markers such as urinary N-acetyl-beta-D-glucosaminidase (uNAG) and kidney injury molecule (uKIM)-1 also entail poor prognosis in CHF.[8,11] Moreover, we have recently shown that when their levels are increasing over time (i.e., when progressive tubular damage [PTD] is present) the association with adverse outcome is even stronger.[8] Importantly, these tubular damage markers predict poor prognosis independently of patients' glomerular function.[8,11]

Taken together it appears that simultaneous biomarker-based monitoring of glomerular and tubular renal compartments carries potential for improvement of renal management of CHF patients during their outpatient follow-up. However, it has not yet been investigated which CHF patients are susceptible to PTD and which to GD. It also remains unclear how these renal biomarkers relate to prognosis when jointly assessed. These considerations are particularly interesting since in current clinical practice tubular damage markers are not routinely assessed, leaving the degree of tubular injury undetermined. Therefore, our aim was to investigate clinical determinants of PTD and GD, and their combined prognostic value for CHF patients.

METHODS

Bio-SHiFT cohort

The Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT) is a prospective cohort of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands. Patients were included if aged ≥18 years and if CHF had been diagnosed ≥3 months ago according to European Society of Cardiology guidelines.[12] Patients were ambulatory and stable, i.e., they had not been hospitalized for HF in the past three months. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients. This investigation comprised 263 stable patients with CHF, who were enrolled during the first inclusion period (October 2011 until June 2013) and completed their follow-up in 2015.

Study visits

All patients were evaluated by research physicians, who collected information on HF-related symptoms, NYHA class, and performed a physical examination and collected samples. Information on HF etiology, ejection fraction, cardiovascular risk factors, comorbidities, and treatment was retrieved from hospital records. Study follow-up visits were predefined and scheduled tri-monthly (±1 month), with a maximum of 10 study follow-up visits. All patients were also routinely followed at the outpatient clinic by treating physicians who were blinded for biomarker data. Occurrence of re-hospitalizations for HF, MI, PCI, CABG, arrhythmias, CVA, cardiac transplantation, left ventricular assist device (LVAD)-placement and mortality was recorded in electronic case-report forms, and associated hospital records and discharge letters were collected. A clinical event committee, blinded for biomarker data, reviewed hospital records and discharge letters and adjudicated the study endpoints.

Study endpoints

The composite endpoint comprised cardiac death, cardiac transplantation, LVAD implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalization for acute

or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with two of the following: BNP or NT-proBNP >3x upper limit of normal, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.[12]

Blood and urine analyses

Samples were collected at baseline and during study visits, and were processed and stored at -80°C. Laboratory personnel was blinded for clinical data. Batch analysis of serum was performed at Erasmus MC: NT–proBNP was analysed using an electrochemiluminesence immunoassay (Roche Diagnostics, Elecsys 2010, Indianapolis, Indiana, USA), cardiac troponin T was also measured using an electrochemiluminesence immunoassay (Roche Diagnostics, Elecsys 2010 immunoassay analyser, Indianapolis, Indiana, USA). Plasma and urine samples were transported at -80°C to HaemoScan BV, Groningen, the Netherlands for batch analysis. Creatinine was determined by a colorometric test by the Jaffé reaction. Plasma was used undiluted, urine was diluted ten times in water (LLD: plasma 0,14 mg/dl, urine: 1.56 mg/ml). KIM-1 was determined in urine diluted 50% in 0,1% BSA/PBS buffer, by ELISA (R&D systems, Minneapolis, MN, USA) (LLD: 0.146 ng/mL). NAG was determined using a substrate p-nitrophenyl N-acetyl-β-D-glucosaminidase at pH 4.5 (Sigma, St Louis, MO, USA) (LLD: 0.485 U/L). All urinary biomarkers were normalized to urinary Cr concentrations to correct for concentration or dilution of urine.The GFR was determined by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation that has been validated in HF patients[13] and categorized using K/DOQI guidelines.[14]

Statistical analysis

To assess patient-specific slopes of renal biomarkers we performed joint modeling (JM) analysis which combines linear mixed-effects (LME) and Cox regression models.[15] The LME models apply a two component equation to construct a biomarker trajectory using its repeated measurements. The first component is a 'fixed-effect' that estimates a biomarker's average trajectory over all patients within the cohort. The second component is a 'random-effect' that estimates by how much an individual patient deviates from this average trajectory at each of study visits during follow-up. By using these two components a patient-specific biomarker trajectory is constructed. Through the random-effects component the LME models allow repeated measurements taken on the same patient to be correlated and incorporates information on the marker's biological variation in each patient (i.e., "noise" around the biomarker

regression trajectory).[16] Finally, JM combines LME and Cox models to adjust biomarker trajectories for different follow-up durations between patients.

From these biomarker trajectories, regression slopes (i.e., rates of biomarker change per year) were calculated which mathematically correspond to the first derivative of a biomarker trajectory.[16] Subsequently, patients were stratified into those in whom no tubular damage marker showed an increased slope, either uNAG or uKIM1 increased, and both markers increased during follow-up. Patients were also stratified into those with increasing Cr levels and those with stable/decreasing Cr levels.

For continuous variables, presence of a linear trend across PTD- and GD-categories was assessed by analysis of variance (ANOVA) or the Kruskal–Wallis test, when appropriate; categorical variables were tested by the $\chi 2$ trend test. Covariates that were univariably associated with PTD or GD (exploratory $p<0.10$) were entered into a multivariable logistic regression model applying proportional odds ordinal regression (for PTD) or binary logistic regression (for GD).

For associations between baseline eGFR and renal biomarkers' slopes, a linear regression analysis was performed using eGFR (per 10 mL/min/1.73m^2) as the independent variable and each of the slopes as the dependent variable on the continuous scale. The models were corrected for the study endpoints; effect heterogeneity of eGFR on study endpoints was tested by adding an interaction term.

To investigate survival rates, we used the two-sided Breslow test and the Breslow method to estimate event-time distributions. Cox regression was performed to assess hazard ratios (HR) with 95% confidence intervals (95%CI) for study endpoints. Statistical adjustments were performed by using biomarker of interest plus age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, eGFR (only for tubular damage markers) and biomarkers of myocardial stretch and damage NT-proBNP and hs-cTnT. Data on all variables were complete, except for systolic blood pressure, which was missing in <5% of patients and for which imputations were applied using patients' clinical and outcome data.

All tests were two-tailed and p-values <0.05 were considered statistically significant. All analyses were performed with SPSS (SPSS 25.0; IBM Corp., Armonk, NY),[17] and R[18] using package JMbayes.[19]

RESULTS

CHF cohort, sample collection and study endpoints

In 263 CHF patients, median age was 67±13 years, 72% were men, 26% were in New York Heart Association (NYHA) functional class III/IV, and 53% had eGFR<60

mL/min/1.73m^2.

During a median of 2.2 (IQR: 1.4–2.5) years, a total of 1984 blood and 1912 urine samples were collected (per patient: 9 [5–10] blood and 8 [5–10] urine samples). Seventy patients (27%) reached the endpoint: 56 patients were re-hospitalized for acute or worsened HF, 9 died of cardiovascular causes, 2 underwent LVAD-placement, and 3 underwent heart transplantation.

Distributions of renal biomarker slopes and their relation to baseline eGFR and study endpoints

Patients who experienced the endpoint had significantly higher slopes of uNAG (mean±SD 0.25±0.30 vs. -0.02±0.27 ln[U/gCr]/year, p<0.001), uKIM1 (0.21±0.36 vs. -0.04±0.24 ln[ng/gCr]/year, p<0.001), and plasma Cr (0.21±0.35 vs. 0.01±0.17 ln[mg/dL]/year, p<0.001) than endpoint-free patients (Figure 1).

When examining baseline eGFR as a continuous variable, eGFR was inversely associated with uNAG and uKIM-1 slopes (i.e., greater PTD was present in patients with lower baseline eGFR), but positively associated with Cr slope (i.e., greater GD was present in patients with higher baseline eGFR). No interactions were found between baseline eGFR and study endpoints (Table 1).

TABLE 1 Slopes of renal biomarkers according to baseline renal function and study endpoints.

Biomarker slopes	β (95% confidence interval)	p-value
uNAG		
Baseline eGFR (per 10 mL/min/1.73m^2 increase)	-0.02(-0.03 to -0.01)	0.030
Study endpoint (yes)	0.26 (0.19 to 0.34)	<0.001
Interaction (eGFR x study endpoint)	**	0.99
uKIM1		
Baseline eGFR (per 10 mL/min/1.73m^2 increase)	-0.02 (-0.03 to -0.01)	0.017
Study endpoint (yes)	0.24 (0.16 to 0.31)	<0.001
Interaction (eGFR x study endpoint)	**	0.69
Creatinine		
Baseline eGFR (per 10 mL/min/1.73m^2 increase)	0.02 (0.01 to 0.04)	<0.001
Study endpoint (yes)	0.21 (0.14 to 0.27)	<0.001
Interaction (eGFR x study endpoint)	**	0.37

** Coefficient not presented since interaction was not significant. Abbreviations: eGFR, estimated glomerular filtration rate; uNAG, urinary N-acetyl-β-D-glucosaminidase; uKIM1, urinary kidney injury molecule 1.

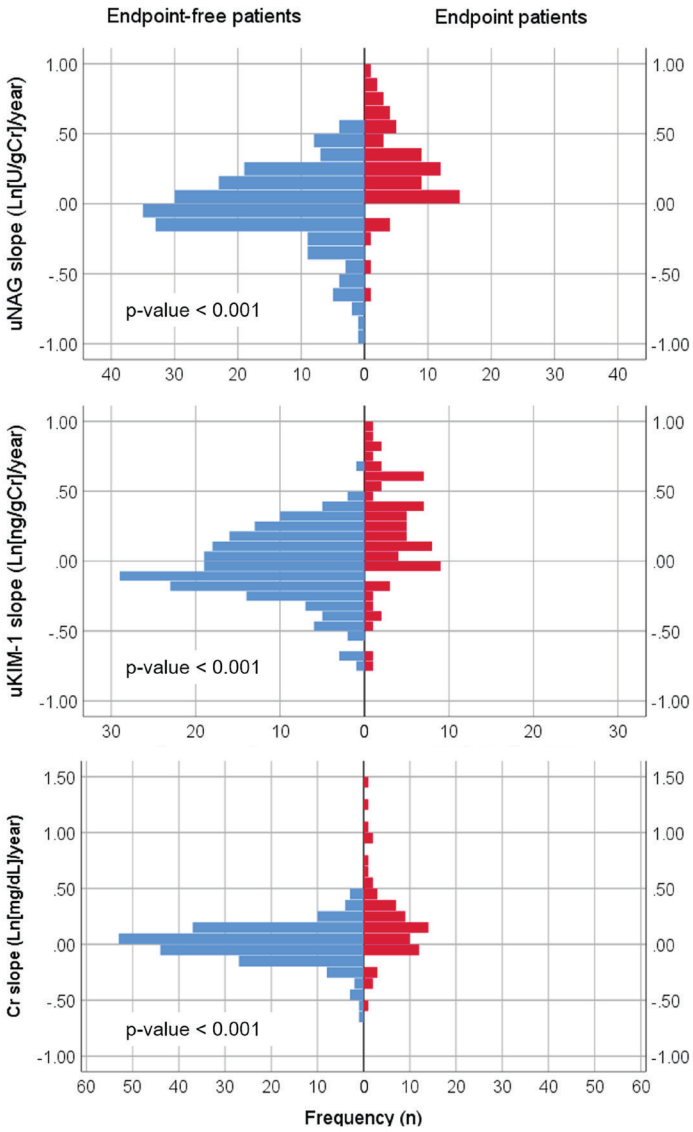


FIGURE 1 Distributions of slopes of renal biomarkers prior to study endpoints. X-axis displays percentage of patients who experienced the event (red) and those who did not (blue), Y-axis displays the estimated slopes on the continuous scale, where positive numbers correspond to increasing slopes and negative numbers correspond to decreasing slopes. T-test was used test the average difference between patient with and without event.

When categorizing patients according to baseline eGFR, we found that patients who experienced the endpoint had higher slopes of all three renal biomarkers than those who did not across all eGFR categories. We also found a tendency towards

more frequent occurrence of PTD and less frequent occurrence of GD in lower eGFR categories (Figure 2).

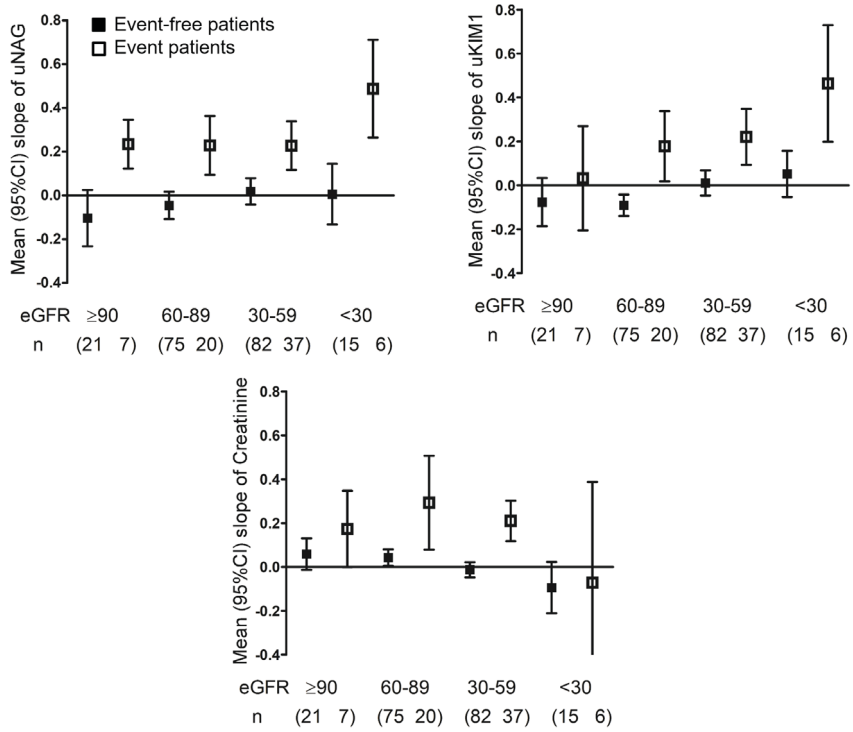


FIGURE 2 Average slopes of renal biomarkers stratified by baseline eGFR category. X-axis displays eGFR categories with absolute number of patients (n), and Y-axis displays the average slopes with 95% confidence intervals, where positive numbers correspond to increasing slopes and negative numbers correspond to decreasing slopes. Black horizontal line depicts stable (zero) slope.

Associations of clinical characteristics with PTD and GD

Seventy five percent of patients (196 of 263) had increasing slope of either uNAG or uKIM1. Of those, both markers were increasing in 43% (85 of 196). Table 2 shows that patients in higher PTD-categories, had higher baseline levels of NT-proBNP, cardiac troponin-T and Cr (eGFR was lower); more frequently diabetes, NYHA class III/IV, and cardiac resynchronization therapy (CRT), and were older. After multivariable adjustments, higher NT-proBNP and lower eGFR levels remained independent clinical predictors of PTD severity (per doubling of NT-proBNP adj. OR 1.26 [95%CI 1.07-1.49], p=0.006; and per 10 mL/min/1.73m^2 eGFR decrease 1.16 [1.03-1.31], p=0.016) (Table 3).

TABLE 2 **Patient characteristics stratified by uNAG and uKIM1 slopes.**

	uNAG&uKIM1 stable/decreased slope (n=67)	uNAG or uKIM1 increased slope (n=111)	uNAG&uKIM1 increased slope (n=85)	p-value
Clinical features				
Age years	65 (57 to 72)	69 (60 to 77)	70 (62 to 79)	0.017*
Men	49 (73)	80 (72)	60 (71)	0.73
HF-rEF	66 (98)	104 (94)	80 (94)	0.24
Ischemic etiology	28 (42)	48 (43)	41 (48)	0.41
BMI kg/m^2	27.1 (25.0 to 30.9)	26.2 (24.1 to 29.0)	26.3 (24.2 to 30.3)	0.55
Heart rate b.p.m.	65 (60 to 74)	66 (60 to 72)	68 (60 to 76)	0.18
SBP mmHg	122 (110 to 135)	120 (106 to 140)	120 (108 to 132)	0.70
DBP mmHg	74 (61 to 82)	73 (65 to 80)	70 (60 to 79)	0.08
Congestion [b]	38 (57)	75 (68)	56 (66)	0.27
NYHA III/IV	9 (13)	28 (25)	32 (38)	0.001*
CRT	27 (41)	35 (32)	18 (21)	0.009*
Medical history				
Prior MI	23 (36)	39 (36)	32 (39)	0.69
Atrial fibrillation	23 (36)	48 (45)	34 (40)	0.65
Diabetes	14 (21)	34 (31)	33 (39)	0.018*
Hypertension	27 (41)	50 (46)	43 (52)	0.18
COPD	8 (12)	10 (9)	13 (16)	0.42
Medication prevalence (%) /average total daily dose (mg)				
Beta-blocker	95/45	91/43	83/47	0.50 [a]
ACE-I/ARBs	96/25	92/25	93/23	0.92 [a]
Loop diuretics	85/77	88/78	96/93	0.35 [a]
MRAs	73/23	68/23	63/23	0.88 [a]
Biomarkers				
NT-proBNP ng/L	592 (158 to 1690)	1196 (448 to 2105)	1650 (857 to 3525)	<0.001*
cTnT ng/L	12.6 (7.5 to 27.2)	17.1 (9.6 to 32.7)	22.4 (13.7 to 43.2)	<0.001*
Glomerular indices				
Creatinine mg/dl	1.10 (0.92 to 1.26)	1.18 (0.97 to 1.43)	1.31 (1.05 to 1.72)	<0.001*
eGFR	70 (48 to 79)	57 (44 to 76)	50 (37 to 71)	<0.001*
eGFR<60	22 (33)	63 (57)	55 (65)	<0.001*
Tubular damage markers				
uNAG, U/gCr	5.2 (2.7 to 10.1)	5.8 (4.0 to 9.1)	6.8 (4.6 to 9.1)	0.22
uKIM1, ng/gCr	447 (235 to 926)	500 (247 to 904)	540 (249 to 994)	0.44

BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; NYHA class, New York Heart Association class; HF-REF, Heart failure with reduced ejection fraction;

eGFR, estimated glomerular filtration rate; MI, myocardial infarction; CVA, cerebrovascular accident; TIA, transitory ischemic attack; COPD, chronic obstructive pulmonary disease; ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers, MRA, mineralocorticoid receptor antagonist; cTnT, cardiac troponin T; CRP, C-reactive protein. eGFR, estimated glomerular filtration rate; uNAG, urinary N-acetyl-β-D-glucosaminidase; uKIM1, urinary kidney injury molecule 1. For reasons of uniformity continuous variables are presented as medians (25th to 75th percentiles) and categorical variables are presented as n (%). p-values signify trend across groups and the asterisk indicates p<0.05.
[a] p-value for the difference in average total daily dose.
[b] Congestion was considered present if ≥2 symptoms or signs were present at baseline (dyspnea, orthopnea, fatigue, elevated jugular venous pressure, presence of rales/crackles and pedal oedema).

TABLE 3 Independent clinical predictors of PTD severity and GD.

	Multivariable model *	
	OR (95% CI)	**p-value**
PTD (dependent variable)[a]		
NT-proBNP (per doubling)	1.26 (1.07-1.49)	p=0.006
eGFR (per 10 mL/min/1.73m^2 decrease)	1.16 (1.03-1.31)	p=0.016
GD (dependent variable)[b]		
Loop diuretics (per 40 mg furosemide dose increase)	1.32 (1.08-1.62)	p=0.006
MRAs (per 25 mg spironolactone dose decrease)	1.76 (1.07-2.89)	p=0.025
eGFR (per 10 mL/min/1.73m^2 increase)	1.40 (1.20-1.63)	p<0.001

OR indicates odds ratio for having GD or more severe PTD; 95%CI, 95% confidence interval for the corresponding OR; GD, glomerular decline; PTD, progressive tubular damage; eGFR, estimated glomerular filtration rate, MRAs, mineralocorticoid receptor antagonists.
[a] Covariates that were found to be different across PTD categories with p<0.10 (Table 2) were entered into a multivariable ordinal regression model, and those were age, diastolic blood pressure, NT-proBNP, hs-cTnT, eGFR, NYHA class, diabetes, use of cardiac resynchronization therapy (CRT).
[b] Covariates that were found to be different between GD and non-GD subgroup with p<0.10 (Table 3) were entered into a multivariable binary regression model, and those were diastolic blood pressure, NT-proBNP, hs-cTnT, eGFR, NAG, prior myocardial infarction, hypertension, atrial fibrillation, loop diuretics and MRAs doses.
* only covariates with p-value <0.05 were presented in the table

Fifty eight percent of patients (153 of 263) had increasing Cr slope. Table 4 shows that these patients had higher baseline levels of NT-proBNP, cardiac troponin-T and uNAG, more frequently had a history of myocardial infarction, and were given higher doses of loop diuretics and lower doses of mineralocorticoid receptor blockers (MRAs). After multivariable adjustments, higher doses of loop diuretics, lower MRA doses, and higher eGFR levels remained independent clinical predictors of GD (per 40 mg increase of furosemide equivalent dose adj. OR 1.32 [1.08-1.62], p=0.006; per 25 mg decrease of spironolactone equivalent dose 1.76 [1.07-2.89], p=0.025; per 10 mL/min/1.73m^2 eGFR increase 1.40 [1.20-1.63], p<0.001) (Table 3).

TABLE 4 **Patient characteristics stratified by creatinine slope.**

	Cr stable/decreased slope (n=110)	Cr increased slope (n=153)	p-value
Clinical features			
Age years	66 (57–75)	69 (60–77)	0.17
Men	74 (67)	115 (75)	0.16
HF-rEF	104 (95)	146 (95)	0.75
Ischemic etiology	45 (41)	72 (47)	0.32
BMI kg/m^2	26.6 (24.2–30.3)	26.4 (24.4–30.1)	0.90
Heart rate b.p.m.	68 (60-77)	66 (60–73)	0.42
SBP mmHg	120 (110–136)	120 (106–132)	0.38
DBP mmHg	75 (66–80)	70 (60-80)	0.07
Congestion	73 (66)	96 (63)	0.55
NYHA III/IV	32 (29)	37 (24)	0.37
CRT	37 (34)	44 (29)	0.40
Medical history			
Prior MI	32 (29)	64 (42)	0.034*
Atrial fibrillation	38 (35)	68 (44)	0.10
Diabetes	29 (26)	52 (34)	0.19
Hypertension	43 (39)	77 (50)	0.07
COPD	9 (8)	22 (14)	0.12
Medication prevalence (%) /average total daily dose (mg)			
Beta-blocker	87 / 48	92 / 42	0.50[a]
ACE-I/ARBs	95 / 22	92 / 26	0.17[a]
Loop diuretics	87 / 62	92 / 97	0.002*[a]
MRAs	69 / 25	67 / 22	0.034*[a]
Cardiac biomarkers			
NT-proBNP ng/L	907 (293–2130)	1406 (520–2804)	0.033*
cTnT ng/L	14.3 (8.5–28.3)	20.6 (10.7–39.1)	0.012*
Glomerular indices			
Creatinine mg/dl	1.29 (1.08–1.63)	1.11 (0.92–1.38)	<0.001*
eGFR	50 (38-70)	63 (48–81)	<0.001*
eGFR<60	71 (65)	69 (45)	0.002*
Tubular damage markers			
uNAG, U/gCr	5.5 (3.4-8.6)	6.6 (4.0–9.4)	0.044
uKIM1, ng/gCr	467.4 (238.3–840.6)	507.6 (247.2–994.1)	0.20

For description please see Table 2; p-values signify a trend across groups. * p<0.05.
[a] p-value for the difference in the average total daily dose.

Study endpoint-free survival and prognosis

Figure 3A displays estimated survival distributions of CHF patients stratified by uNAG and uKIM1 slopes. Survival rates were lowest when both biomarkers were increased, followed by survival rates when either marker's slope was increased (p for trend <0.001). Hazard ratios were significantly higher as compared to the category of patient in whom both markers were stable or decreasing during follow-up (uNAG or uKIM-1 slope increased: adj. HR 4.2 [95%CI: 1.2-13.9], p=0.021; uNAG & uKIM-1 slopes increased: 8.1 [2.4-26.6], p=0.001). These estimates were independent of the patients' clinical characteristics, baseline eGFR, NT-proBNP, and cardiac troponin T.

In Figure 3B, patients with increasing Cr slope had lower survival rates than their counterparts (p=0.012). The hazard in these patients was also significantly higher and independent of patients' clinical characteristics, NT-proBNP, and cardiac troponin T (Cr slope increased: HR 1.9 [1.1-3.3], p=0.025).

Figure 4 displays the Kaplan-Meier curves of patients stratified by uNAG, uKIM1, and Cr. The figure shows that when the slopes of tubular damage markers were stable or improving, glomerular decline did not affect survival rates. However, if either uNAG or uKIM1 slope increased, the survival rates decreased. Finally, the lowest survival rates were in patients who had increasing slopes of all three renal biomarkers (p for trend <0.001).

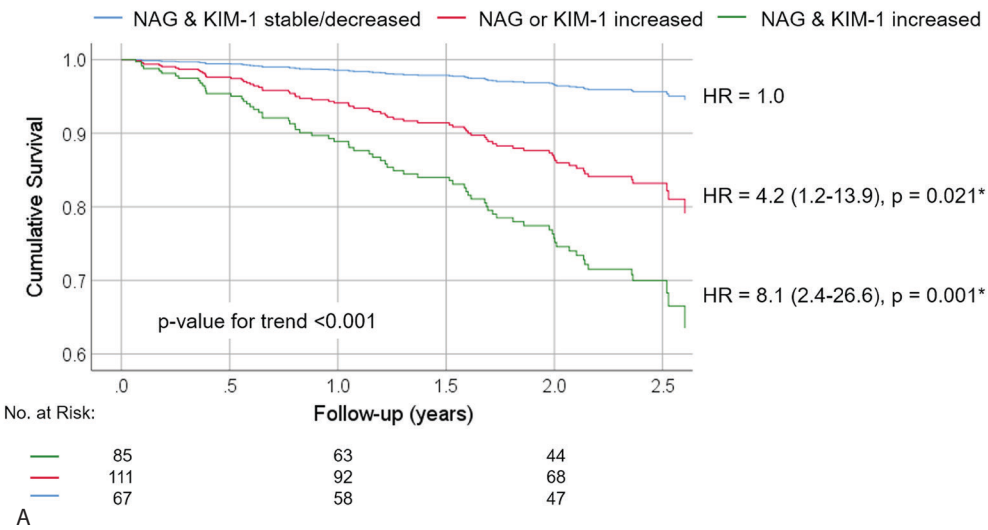
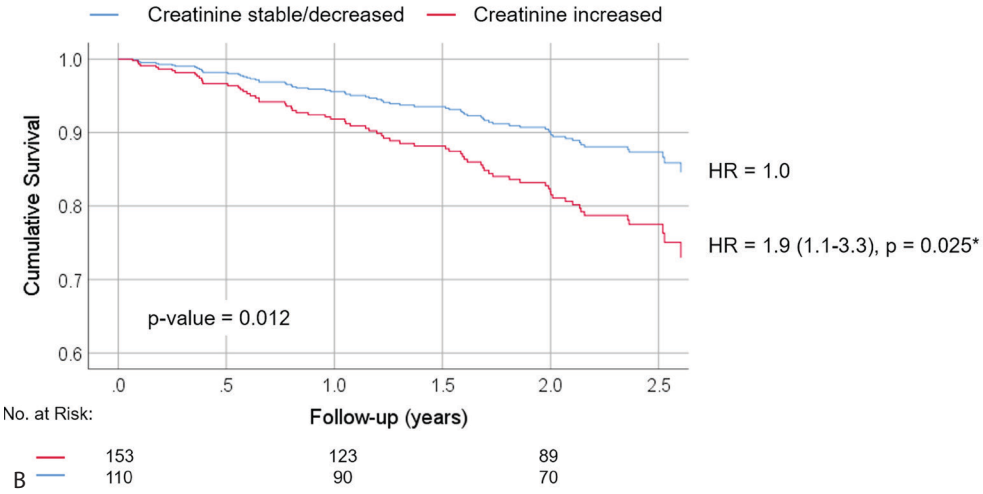


FIGURE 3 Kaplan-Meier Survival Curves stratified by slopes of renal biomarkers. Shown are Kaplan-Meier (KM) curves for the cumulative event-free survival of the composite of HF-rehospitalization, cardiac death, LVAD placement, and heart transplantation. **A**. KM curves are stratified by whether both uNAG and uKIM1 slopes were



decreasing/stable (blue); either uNAG or uKIM1 slope was increasing (red); or both uNAG and uKIM1 slopes were increasing (green); **B.** KM curves are stratified by whether creatinine slope was decreasing/stable (blue) or increasing (red). *adjusted for age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, eGFR (only for tubular damage biomarkers), NT-proBNP, and hs-cTnT.

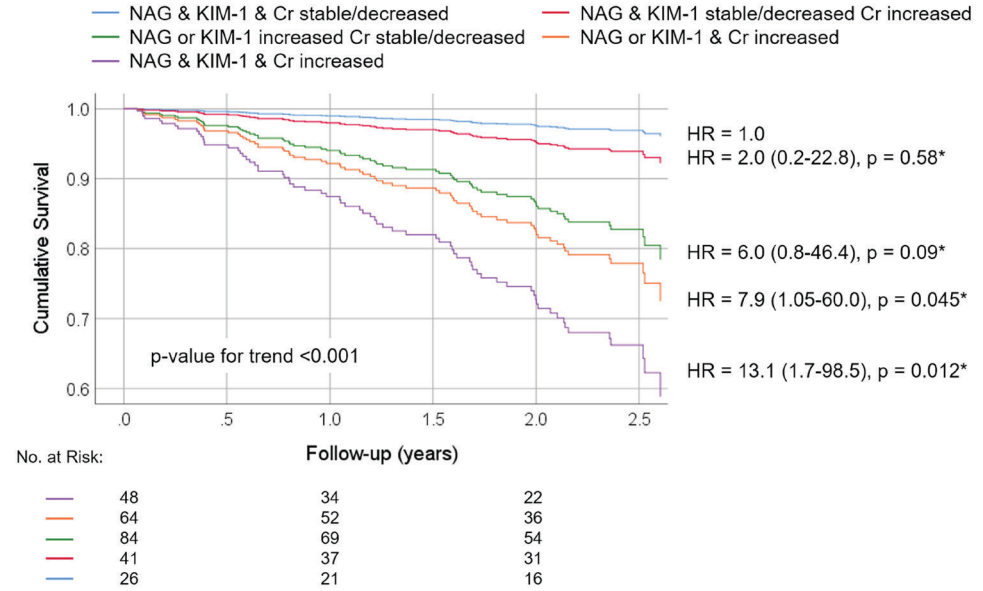


FIGURE 4 Kaplan-Meier Survival Curve stratified by combined slopes of renal biomarkers. KM curves are stratified by whether slopes of all three renal biomarkers were decreasing/stable (blue); uNAG and uKIM1 slopes were decreasing/stable, but creatinine (Cr) slope was increasing (red); either uNAG or uKIM1 slope was increasing but creatinine slope was decreasing/stable (green); either uNAG or uKIM1 slope was increasing, and Cr slope was

increasing (orange); and slopes of all three biomarkers were increasing (purple). *adjusted for age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, NT-proBNP, and hs-cTnT.

DISCUSSION

This study is the first to assess combined effects of PTD and GD on clinical endpoint-free survival during outpatient follow-up of patients with CHF. We show that patients in whom both renal compartments deteriorate over time have the lowest endpoint-free survival. Conversely, the highest endpoint-free survival was observed in patients without signs of PTD, regardless of their Cr slope pattern. To our best knowledge, this is also the first study to identify clinical predictors of PTD severity in CHF. Of note, these determinants differ from those found in GD, which strengthens the recommendation that glomerular and tubular damage markers should be jointly assessed.

Renal function may act as a barometer of cardiac function in CHF. [20] However, because of the multi-factorial nature of cardiorenal interactions, merely assessing the glomerular filtration rate of the kidney may be suboptimal for decision-making. Our study confirms this, and provides an additional evidence that the failing heart affects glomerular and tubular compartments differently over time. In this study, one of the striking findings is that the change in tubular markers may be even clinically more relevant than the change in Cr. Importantly, the rates of change in each aspect of the kidney (glomerular and tubular) provide incremental prognostic information, and together may further identify higher-risk individuals and herewith improve clinical monitoring of CHF patients. These kidney-specific signals may, therefore, help physicians to better, and timely, target medical therapy before the future event occurs. It could also be speculated that "renoprotective" treatment targeted at the tubules may be even more effective than treatment aiming at improving renal function in terms of GFR by means of afferent/efferent vasodilating agents. However, interventional studies on these tubular damage markers are needed to provide definite answers in this matter.

In patients who had PTD, we found lower baseline eGFR. This suggests that patients who had fewer functioning nephrons, were more susceptible to tubular deterioration. This may be attributed to work-overload in residual nephrons to compensate renal function.[21] Despite the loss in total GFR, compensatory hyperfiltration in these nephrons may exceed tubular capacity leading to their progressive damage. These patients more frequently had diabetes, which may also have contributed to PTD. Similarly, other clinical determinants such as aging kidneys and severity of HF (higher cardiac markers, NYHA class, and CRT) indicate that

factors that are related to more severe HF, also cause tubule-specific kidney damage. Importantly, our findings suggest that simultaneous assessment of both uNAG and uKIM-1 translates into better risk stratification of patients than assessment of either one alone. Importantly, these biomarkers predicted poor survival even in patients with apparently stable glomerular function during outpatient follow-up.

GD was found to be associated with higher baseline eGFR which is supported by several previous studies.[22-24] However, this finding is inconsistent with the general opinion that GD (defined as worsening renal function [WRF] with delta-Cr >0.03mg/dl) occurs more frequently in CHF patients that have impaired GFR already at baseline.[25] However, has also been reported that when studies defined WRF as eGFR change instead of Cr change,[23,24] paradoxically, the patients with WRF had lower baseline Cr levels. Interestingly, in the studies that reported lower baseline Cr levels in patient with GD, average baseline Cr was 1.15 mg/dl,[22-24] whereas in the studies that reported higher baseline Cr levels in patients with GD, average baseline Cr was 1.41 mg/dl (average of all reported values in CHF cohorts on WRF).[1] Thus, it seems that studies in which baseline renal impairment was associated with GD recruited patients with worse baseline renal function than those in which the opposite was found. Furthermore, the dissimilar degree of tubular damage could have affected this relationship, as higher tubular damage relates to glomerular decline. However, a definite answer cannot be given because many studies lack these data. Moreover, closer monitoring of patients who already had impaired GFR could have also increased the likelihood of finding WRF in these patients,[26] and particularly if sampling was not fixed but left at the discretion of the treating physician.[27] Finally, a "regression to the mean" could also account for observed discrepancies. As for our study, the observations were made using more than twice as many repeated measurements as in each of the previous studies, samples were collected at fixed time intervals, and the treating physicians were unaware of biomarker data. This further strengthens our suggestion that GD should not be disregarded in CHF patients with relatively intact GFR. Finally, higher doses of loop diuretics and lower MRA doses were identified in glomerular decliners and are supported by previous studies. [1,26]

Study limitations

Several limitations merit consideration. First, this study lacked direct GFR measurement. Second, we cannot comment on the effects of glomerular permeability on clinical outcome since we did not measure proteinuria. Third, although trials on this subject are lacking, and causal inference is limited by the observational nature of our study, the repeated-measures design of this study allows for stronger claims of true associations than previous studies do.

CONCLUSION

Progressive tubular damage and glomerular decline are coupled with different clinical profiles of CHF patients, and those in whom both renal compartments deteriorated had the poorest prognosis. Slopes of urinary tubular damage markers uNAG and uKIM-1 appear to be clinically important even without concomitant glomerular decline, which is of particular interest since in current clinical practice these markers are not routinely assessed and the degree of tubular injury remains undetermined.

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CHAPTER 6

Utility of Temporal Profiles of new Cardio-renal and Pulmonary Candidate Biomarkers in Chronic Heart Failure

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ABSTRACT

Background

Our aim was to explore potential use of temporal profiles of seven emerging cardio-renal and two pulmonary candidate biomarkers for predicting future adverse clinical outcome in stable patients with chronic heart failure (CHF).

Methods

In 263 CHF patients, we determined the risk of a composite endpoint of HF-hospitalization, cardiac death, LVAD-placement and heart transplantation in relation to repeatedly assessed (567 samples in total) blood biomarker levels, and slopes of their temporal trajectories (i.e., rate of biomarker change per year). In each patient, we estimated biomarker trajectories using repeatedly measured osteopontin (OPN), osteoprotegerin (OPG), epidermal growth factor receptor (EGFR), heparin-binding protein (HBP), trefoil factor-3 (TFF3), kallikrein-6 (KLK-6), matrix extracellular phosphoglycoprotein (MEPE), pulmonary surfactant-associated protein-D (PSP-D), and secretoglobulin family 3A-member-2 (SCGB3A2).

Results

During 2.2 years of follow-up, OPN, OPG, and HBP levels predicted the composite endpoint (univariable hazard ratio [95% confidence interval] per 1SD increase: 2.31 [1.76-3.15], 2.23 [1.69–3.00], and 1.36 [1.09-1.70]). Independently of the biomarkers' levels, the slopes of OPG, TFF-3, PSP-D trajectories were also strong clinical predictors (per 0.1SD increase/year: 1.24 [1.14–1.38], 1.31 [1.17–1.49], and 1.32 [1.21–1.47]). All associations persisted after multivariable adjustment for baseline characteristics, and repeatedly assessed CHF pharmacological treatment and cardiac biomarkers NT-proBNP and troponin T.

Conclusions

Repeatedly-measured levels of OPN, OPG, and HBP, and slopes of OPG, TFF-3, and PSP-D strongly predict clinical outcome. These candidate biomarkers may be clinically relevant as they could further define a patient's risk and provide additional pathophysiological insights into CHF.

INTRODUCTION

Chronic heart failure (CHF) is a clinical syndrome which often requires constant therapeutic interventions due to recurrent episodes of cardiac decompensation.[1] The failing heart also induces structural and functional changes in distant organs such as the kidneys and the lungs.[2,3] Eventually, a vicious circle of pathophysiological processes is formed between these organs leading to end-stage heart failure.[4,5] In this context, circulating biomarkers that reflect the status of this multi-organ pathophysiology may be a valuable clinical tool, as these biological signals precede decompensation and may provide early organ-specific information in CHF. Therefore, patient-specific biomarker profiles may further characterize the multi-organ involvement in CHF, but may also help in monitoring disease progression to allow timely adaptation of treatment to prevent impending decompensation.

Although previous biomarker-based studies have increased our understanding of CHF[6,7], several important aspects of biological signals in CHF remain to be addressed. Most previous studies have examined the prognostic value of a single baseline assessment which is unable to capture progression of CHF that naturally occurs over time. These studies also used conventional statistical models that do not allow for individualized risk prediction using patient-specific biomarker values and their change over time. Finally, similar sets of CHF biomarkers have been investigated by most of the existing studies such as natriuretic peptides, troponins, and markers representing certain aspects of CHF like galectin-3 and ST2.

Data on the utility of new candidate biomarkers in CHF are scarce, and their clinical value remains uncertain. Therefore, in this study, our aim was to explore the prognostic utility of temporal profiles of several emerging cardio-renal and pulmonary candidate biomarkers in CHF patients during their outpatient follow-up.

Cardio-renal candidate biomarkers included osteopontin (OPN), which is associated with accumulation of monocytes/macrophages in injured renal tissues including both glomeruli and tubules,[8] and which is mainly overexpressed in cardiac non-myocytes during pathological cardiac remodeling;[9] osteoprotegerin (OPG), which is involved in bone metabolism, endocrine function, and immunity,[10] and is secreted mainly by osteoblasts and by vascular smooth muscle and endothelial cells, but also in the renal tissue;[11] matrix extracellular phosphoglycoprotein (MEPE), which is another molecule that regulates bone metabolism, and in particular phosphates handling in the renal tubules;[12] trefoil factor-3 (TFF3), which is a member of the trefoil factor peptide family secreted by the renal tubulocites in response to injury;[13] heparin-binding protein (HBP), which is released from neu-

trophils upon activation, after which it induces vascular leakage, edema formation, and inflammatory reactions which play a role in sepsis-induced acute kidney injury (AKI);[14-16] epidermal growth factor receptor (EGFR), which is a tyrosine kinase receptor found to be involved in acute and chronic renal injury;[17] and kallikrein 6 (KLK-6) which is a recently identified member of the kallikrein gene family and is involved in degradation of extracellular matrix during tumor invasion and metastasis, but also in demyelization and spinal cord injury.[18,19]

Pulmonary candidate biomarkers included pulmonary surfactant-associated protein-D (PSP-D), which was found to reduce alveolar macrophages apoptosis and to promote clearance of necrotic cells after lung injury,[20] and secretoglobulin family 3A-member-2 (SCGB3A2), which is another newly discovered biomarker with prominent anti-inflammatory and anti-fibrotic activity in animal models of pulmonary fibrosis.[21]

METHODS

CHF cohort

The Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (BioSHiFT) is a prospective cohort of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands.[22,23] Patients were included if aged ≥18 years, capable of understanding and signing informed consent, and if CHF had been diagnosed ≥3 months ago according to European Society of Cardiology guidelines.[1,24,25] Patients were ambulatory and stable, i.e., they had not been hospitalized for HF in the past three months. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients. This investigation comprised 263 CHF patients enrolled during the first inclusion period (October 2011 until June 2013).

Baseline and follow-up assessment

All patients were evaluated by research physicians, who collected information on HF-related symptoms, NYHA class, and performed a physical examination. Information on HF etiology, left ventricular ejection fraction (EF of 50% at inclusion used as a cut-off for HFrEF versus HFpEF)[25], cardiovascular risk factors, medi-

cal history and treatment was retrieved primarily from hospital records and was checked in case of ambiguities.

During the study, all patients were routinely followed at the outpatient clinic by their treating physicians. Additionally, study follow-up visits were predefined and scheduled every 3 months (±1 month). At each study follow-up visit, a short medical evaluation was performed and blood and urine samples were collected. During follow-up, all medication changes and occurrence of hospitalizations for HF, MI, PCI, CABG, arrhythmias, and CVA, cardiac transplantation, left ventricular assist device (LVAD) implantation and mortality, were recorded in the electronic case report forms, and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

Study endpoints

The composite endpoint comprised of hospitalization for the management of acute or worsened HF, cardiac death, cardiac transplantation, and LVAD implantation, whichever occurred first. Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalization for acute or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with two or more of the following: BNP or NT-proBNP >3x upper limit of normal, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.[24]

Study measurements and laboratory analysis

Blood samples were collected at baseline and at each 3-monthly study follow-up visit, and were processed and stored at -80°C within two hours after collection. Treating physicians were unaware of biomarker results as biomarkers were measured batchwise after completion of follow-up. All laboratory personnel was blinded for clinical data and patients outcomes. Batch analysis of serum was performed at Erasmus MC: NT–proBNP was analysed using an electrochemiluminesence immunoassay (Roche Diagnostics, Elecsys 2010, Indianapolis, Indiana, USA) and cardiac troponin T was also measured using an electrochemiluminesence immunoassay (Roche Diagnostics, Elecsys 2010 immunoassay analyser, Indianapolis, Indiana, USA). Plasma samples were transported at a temperature of -80°C to

HaemoScan BV, Groningen, The Netherlands where creatinine was determined by a colorometric test by the Jaffé reaction.

Thus, the biomarker measurements did not lead to drug adjustments. All patients received treatment according to the ESC guidelines on CHF.[1,24] For efficiency, for the current investigation we selected all baseline samples, the two samples closest in time to the composite endpoint, and for patients in whom the primary endpoint did not occur during follow-up, the last sample available. Glomerular filtration rate (GFR) was determined by the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation validated in HF patients.[26]

The Olink multiplex PEA platform panel for new biomarkers

The Olink Cardiovascular (CVD) panel III was used for analysis of high-abundance proteins (Olink Proteomics AB, Uppsala, Sweden). The proteins present in this Olink panel were selected because either they have a proven pathophysiological role in cardiovascular disease, or because they are promising in this respect but yet unexplored. In the current investigation, biomarkers from the panel were chosen and grouped based on their previously described predominant tissue expression and involvement in renal[9,16,27-31] and/or pulmonary[32,33] pathophysiology.

The Olink panel is based on PEA (proximity extension assay) technology[34] which uses two oligonucleotide-labeled antibodies to bind to their respective target proteins in the sample. When the two antibodies are in close proximity, a new PCR target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using standard real-time PCR. Each sample includes two incubations, one extension, and one detection control to determine the lower limit of detection and normalize the measurements.The biomarkers are presented in normalized protein expression (NPX) units on a 2log scale. In a validation study, the mean intra-assay and inter-assay coefficients of variation were 8% and 12%, respectively.[34]

Statistical analysis

For the analysis, we used the Z-score (i.e., the standardized form) of the 2log-transformed biomarkers to allow for direct comparisons of different biomarkers. We used a network analysis[35] to assess the relationships between biomarkers with Pearson's correlation coefficients $p<0.05$ using a clustering coefficient as a measure of the degree to which biomarkers tend to cluster together (higher coefficients suggest a certain centrality of a biomarker within the network).[36]

To study the effect of baseline characteristics on repeatedly measured biomarkers, linear mixed-effects (LME) models were performed using biomarkers as the dependent variables and baseline characteristics as the independent variables (fixed part). The sampling time was entered into the fixed- and random parts of the models.

To estimate the associations between biomarker levels and survival, we applied a joint modeling (JM) prediction analysis that combines LME models for repeated measurements, and Cox survival analysis for time-to-event data.[37] For both the fixed- and random-effects parts of the LME models, linear terms were used for sampling times, and both intercepts and slopes were included in the random-effects design matrix. This allowed the markers' trajectories to differ at baseline and over time. We also estimated the time-dependent slope (i.e., rate of change) of each biomarker, indicating whether and by how much the levels are increasing or decreasing on a continues scales.

Besides sampling time, all markers were adjusted as follows: (1) clinical model: Cox and LME models were adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and eGFR; (2) clinical & time-varying HF medication model: after adjusting for clinical characteristics, biomarker values were extracted from the joint models and entered simultaneously with repeatedly assessed equivalent doses of carvedilol, enalapril, furosemide, and spironolactone into a time-dependent Cox analysis to examine the incremental value of the new biomarkers over clinical characteristics and medication during follow-up; (3) time-dependent Cox model using the marker's fitted values adjusted for type of HF (HFrEF vs. HFpEF), and time-varying NT-proBNP and hs-cTnT collected at the same time points during follow-up as the biomarker of interest. Data on all variables were complete, except for systolic blood pressure which was missing in <5% of patients and for which imputations were applied using the patients' clinical and outcome data. Results are given as hazard ratios (HR) and 95% confidence intervals (CI) per 1SD increase of the marker's level and per 0.1SD increase of the slope at any time-point during follow-up.

To correct for multiple testing, we performed matrix spectral decomposition which has been used in genetic studies as it has been demonstrated to be more effective than Bonferroni correction.[38] In this way, we accounted for the correlations between the biomarkers by setting a significance level at $p < 0.008$ (0.05/6).

All tests were two-tailed and were performed with R Statistical Software using packages nlme and JMbayes.[37] The network analysis was performed using Gephi software (https://gephi.org) and the matSpD application (https://gump.qimr.edu.au/general/daleN/matSpD) available online.

RESULTS

Baseline characteristics

Patients who experienced the primary endpoint during follow-up were older, more frequently had diabetes, atrial fibrillation, lower systolic blood pressure, higher NYHA class, higher levels of NT-proBNP and cardiac troponin T, and were more frequently on diuretics (Table 1). All biomarkers showed significantly higher levels at baseline, except for EGFR which was lower, in patients who later experienced the endpoint than in endpoint-free patients (Figure S1).

TABLE 1 Patients characteristics in relation to the composite endpoint.

Variable	Total	Composite endpoint		p-value
		Yes	No	
N (%)	263 (100)	70 (27)	193 (73)	
Demographics				
Age, years	67±13	69±13	66±12	0.05
Men, n (%)	189 (72)	53 (76)	136 (70)	0.41
Clinical characteristics				
BMI, kg/m^2	27.5±4.7	27.6±4.8	27.4±4.7	0.80
Heart rate, b.p.m.	67±12	69±13	67±11	0.31
SBP, mmHg	122±20	117±17	124±21	0.02
DBP, mmHg	72±11	70±10	73±11	0.06
Features of heart failure				
NYHA class III /IV, n (%)	69 (26)	31 (44)	38 (20)	< 0.001
HF-rEF n (%)	250 (95)	66 (94)	184 (95)	0.75
HF-pEF n (%)	13 (5)	4 (6)	9 (5)	
LVEF, %	32±11	30±11	33±10	0.18
NT pro-BNP (ng/L) †	1161 (439-2305)	2388 (1492–4376)	806 (268–1757)	< 0.001
Hs-TnT (ng/L) †	18.0 (9.5–33.2)	31.9 (20.6–49.7)	13.9 (8.4–26.7)	< 0.001
Etiology of heart failure, n (%)				
Ischemic	117 (44)	36 (51)	81 (42)	0.17
Hypertension	34 (13)	10 (14)	24 (12)	0.70
Valvular disease	12 (5)	5 (7)	7 (4)	0.23
Cardiomyopathy	68 (26)	15 (21)	53 (28)	0.32
Unknown or Others	32 (12)	4 (6)	28 (15)	
Medical history, n (%)				
Prior MI	96 (36)	32 (46)	64 (33)	0.06

continued

Variable	Total	Composite endpoint		p-value
		Yes	No	
Prior PCI	82 (31)	27 (39)	55 (28)	0.12
Prior CABG	43 (16)	13 (19)	30 (15)	0.57
Atrial fibrillation	106 (40)	36 (51)	70 (36)	0.03
Diabetes	81 (31)	32 (46)	49 (25)	0.002
Hypercholesterolemia	96 (36)	30 (43)	66 (34)	0.20
Hypertension	120 (46)	38 (54)	82 (42)	0.09
COPD	31 (12)	12 (17)	19 (10)	0.10
Medication use, n (%)				
Beta-blocker	236 (90)	61 (87)	175 (91)	0.40
ACE-I or ARB	245 (93)	63 (90)	182 (94)	0.22
Diuretics	237 (90)	68 (97)	169 (88)	0.02
Loop diuretics	236 (90)	68 (97)	168 (87)	0.02
Thiazides	7 (3)	3 (4)	4 (2)	0.28
AA	179 (68)	53 (76)	126 (65)	0.11
Glomerular function				
Creatinine, mg/dl	1.18 (0.99–1.49)	1.30(1.02–1.52)	1.17(0.98–1.45)	0.18
eGFR, mL/min/1.73m^2	58 (43–76)	53 (40–73)	59 (44–77)	0.16
KDOQI classification, n (%)				0.18
eGFR ≥90	28 (11)	7 (10)	21 (11)	
eGFR 60-89	95 (36)	20 (28)	75 (39)	
eGFR 30-59	119 (45)	37 (53)	82 (42)	
eGFR <30	21 (8)	6 (9)	15 (8)	

BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; NYHA class, New York Heart Association class; HF-rEF, Heart failure with reduced ejection fraction; HF-pEF, heart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction; MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; CVA, cerebrovascular accident; TIA, transitory ischemic attack; COPD, chronic obstructive pulmonary disease; ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; AA, aldosterone antagonist; eGFR, estimated glomerular filtration rate.
†Median with inter-quartile range (IQR). Normally distributed continuous variables are presented as mean ± standard deviation (SD), and non-normally distributed variables as median and interquartile interval. Categorical variables are presented as numbers and percentages.

Follow-up and study endpoints

During a median (IQR) follow-up of 2.2 (1.4–2.5) years, we collected at fixed 3-month intervals a median (IQR) of 9 (5–10) blood samples per patient (1984 samples in total). Seventy (27%) patients reached the composite endpoint: 56 patients were re-hospitalized for acute or worsened HF, 3 patients underwent heart transplantation, 2 patients underwent LVAD placement, and 9 patients died of cardiovascular causes. For reasons of efficiency, we set out to select all baseline samples, the two samples closest in time to the composite endpoint, and the last sample available for event-free patients for biomarker measurement. Some of these samples were not available, for example in case an endpoint occurred early after baseline or before next scheduled study visit. Ultimately, 567 samples were used for biomarker measurement.

Patients' clinical profile and biomarkers during follow-up

Table 2 shows the associations between the patients' baseline clinical profiles and the repeatedly-measured levels of candidate biomarkers during follow-up. Furthermore, we found a negative association between time-varying enalapril equivalent doses and OPN, OPG, PSP-D, and SCGB3A2 levels during follow-up (Table S1). Moreover, a negative association was observed between spironolactone equivalent doses and OPG, KLK-6, PSP-D, and SCGB3A2 levels, whereas furosemide equivalent doses correlated positively with OPN, TFF-3, KLK-6, and MEPE levels during follow-up.

Network analysis

The network analysis showed that OPN and TFF3 had the highest clustering coefficients which suggests that these two biomarkers had a certain centrality within the network, meaning that a large number of biomarker correlations are mediated thought these hubs (Figure 1).

Temporal trends in biomarkers and relation to study endpoint

Figure 2 shows the average temporal evolutions of candidate biomarkers in patients who reached the composite endpoint and those who remained endpoint-free. In patients who reached the endpoint, OPN, OPG, HBP, and TFF3, PSP-D, and SCGB3A2 showed higher baseline levels that increased further during follow-up as the endpoint approached. Patients with the endpoint also had constantly higher

levels of KLK-6 and MEPE, but without a further increase in the approach to the endpoint. Table 3 shows the associations of these biomarkers with the composite endpoint.

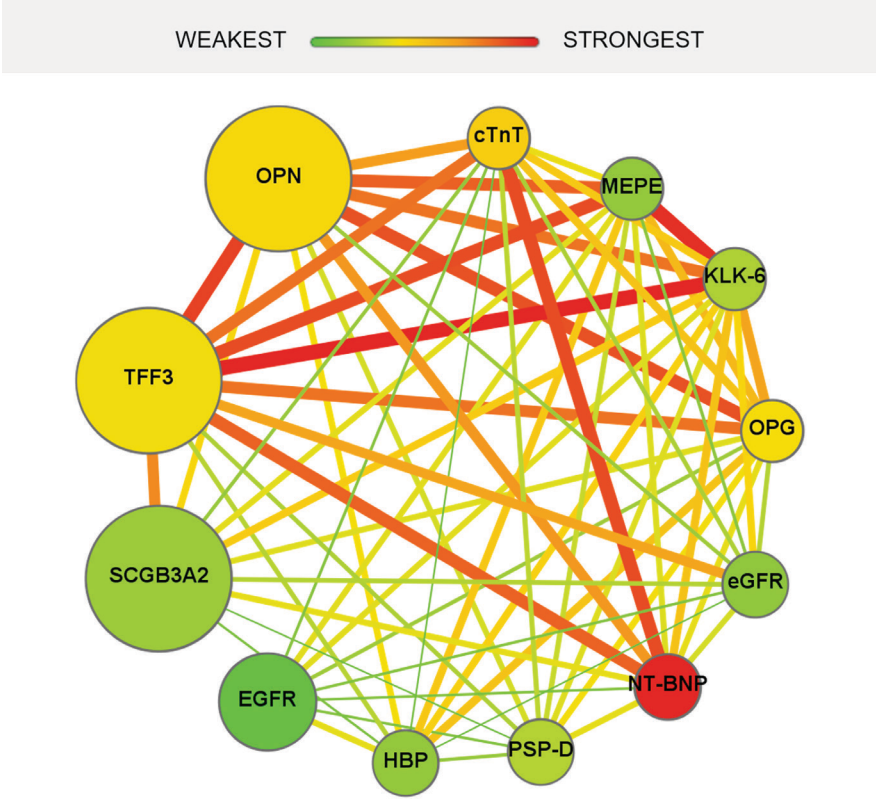

FIGURE 1 Network analysis of candidate biomarkers depicting inter-marker correlations and associations with the composite outcome. Node color displays crude association with primary outcome, and ranges from the weakest (green) to the strongest (red); node size displays clustering coefficient (a measure of the degree to which biomarkers tend to cluster together suggesting a certain centrality within the biomarker network). Thickness of the line between the biomarkers and line color represent the correlation coefficient; correlation coefficient is presented only if p-value <0.05. A ticker line represents stronger coefficients and line color ranges from the weakest (green) to the strongest (red). OPN, osteopontin; OPG, osteoprotegerin; EGFR, epidermal growth factor receptor; HBP, heparin-binding protein; TFF3, trefoil factor 3; PSP-D, pulmonary surfactant-associated protein D; SCGB3A2, secretoglobulin family 3A member 2; KLK-6, kallikrein-6; MEPE, matrix extracellular phosphoglycoprotein.

TABLE 2 **Association of baseline features with repeatedly measured levels of candidate biomarkers during follow-up.**

Independent variable	Dependent variable									
	OPN		OPG		EGFR		HBP		TFF3	
	β(95% CI)	p-value	β(95% CI)	p-value	β(95% CI)	p-value	β(95% CI)	p-value	β(95% CI)	p-value
Age per 10 yrs.		ns		ns	-0.29 (-0.38 to -0.20)	<0.001		ns		ns
Male sex		ns	-0.25 (-0.47 to -0.04)	0.022		ns		ns	-0.27 (-0.46 to -0.08)	0.006
NYHA class per 1 point increase		ns		ns	-0.16 (-0.28 to -0.04)	0.009		ns	0.20 (0.08 to 0.31)	0.001
DM		ns	0.27 (0.05 to 0.48)	0.015		ns		ns		ns
AF	0.28 (0.10 to 0.47)	0.003	0.37 (0.18 to 0.58)	<0.001		ns	0.23 (0.03 to 0.44)	0.026		ns
SBP per 10 mmHg		ns		ns		ns		ns		ns
eGFR per 20 mlmin/1.73m^2		ns	-0.12 (-0.22 to -0.02)	0.015		ns	-0.10 (-0.20 to -0.01)	0.038	-0.21 (-0.30 to -0.13)	<0.001
NT-proBNP per doubling	0.10 (0.04 to 0.16)	<0.001		ns		ns		ns	0.13 (0.07 to 0.18)	<0.001
cTnT per doubling	0.18 (0.07 to 0.28)	<0.001	0.12 (0.01 to 0.23)	0.033		ns		ns	0.17 (0.07 to 0.26)	0.001
Carvedilol eqv. per 50 mg		ns		ns		ns		ns		ns
Enalapril eqv. per 40 mg		ns	-0.21 (-0.38 to -0.04)	0.015		ns		ns		ns
Furosemide eqv. per 40 mg	0.06 (0.04 to 0.09)	<0.001		ns		ns	0.06 (0.02 to 0.11)	0.009	0.06 (0.02 to 0.11)	0.002
Spironolactone eqv. per 25 mg	-0.16 (-0.29 to -0.02)	0.03		ns		ns	-0.21 (-0.37 to -0.05)	0.012		ns

continued

Independent variable	Dependent variable							
	KLK-6		MEPE		PSP-D		SCGB3A2	
	β(95% CI)	p-value	β(95% CI)	p-value	β(95% CI)	p-value	β(95% CI)	p-value
Age per 10 yrs.		ns		ns		ns		ns
Male sex		ns		ns		ns	-0.41 (-0.66 to -0.16)	0.002
NYHA class per 1 point increase		ns		ns		ns	0.19 (0.04 to 0.34)	0.013
DM		ns		ns		ns	-0.46 (-0.71 to -0.22)	<0.001
AF		ns		ns		ns		ns
SBP per 10 mmHg	-0.05 (-0.10 to 0.00)	0.047	-0.06 (-0.11 to 0.00)	0.041		ns		ns
eGFR per 20 mlmin/1.73m^2	-0.23 (-0.33 to -0.14)	<0.001	-0.16 (-0.27 to -0.06)	0.002		ns		ns
NT-proBNP per doubling	0.08 (0.02 to 0.14)	0.007		ns	0.09 (0.02 to 0.17)	0.016		0.039
cTnT per doubling	0.21 (0.10 to 0.31)	<0.001	0.19 (0.06 to 0.31)	0.003		ns		ns
Carvedilol eqv. per 50 mg		ns	0.14 (0.01 to 0.27)	0.038		ns		ns
Enalapril eqv. per 40 mg		ns		ns		ns		ns
Furosemide eqv. per 40 mg		ns	0.06 (0.01 to 0.11)	0.016		ns		ns
Spironolactone eqv. per 25 mg	-0.43 (-0.59 to -0.28)	<0.001	-0.19 (-0.36 to -0.01)	0.034	-0.24 (-0.43 to -0.05)	0.013		ns

OPN, osteopontin; OPG, osteoprotegerin; EGFR, epidermal growth factor receptor; HBP, heparin-binding protein; TFF3, trefoil factor 3; PSP-D, pulmonary surfactant-associated protein D; SCGB3A2, secretoglobulin family 3A member 2; KLK-6, kallikrein-6; MEPE, matrix extracellular phosphoglycoprotein.
The effects of patients' baseline characteristics are given as adjusted β (95% confidence interval) for 1SD differences of biomarkers as measured on the 2log scale. This method allows a direct comparison of the effects on different biomarkers. All βs are adjusted for age, sex, diabetes mellitus (DM), atrial fibrillation (AF), baseline NYHA class, systolic blood pressure (SBP), estimated glomerular filtration rate (eGFR), NT-proBNP levels, cardiac troponin T levels (cTnT), and equivalent doses of carvedilol, enalapril, furosemide, and spironolactone. Only the associations with significance level of p-value <0.05 are presented.

Osteopontin [NPX]
A
Osteoprotegerin [NPX]
B
Epiderma growth factor Rc [NPX]
C
Heparin-binding protein [NPX]
D
Trefoil factor 3 [NPX]
E
Kallikrein-6 [NPX]
F
Event pts.
Event-free pts.
NPX
BL
Months from baseline
Months until the endpoint

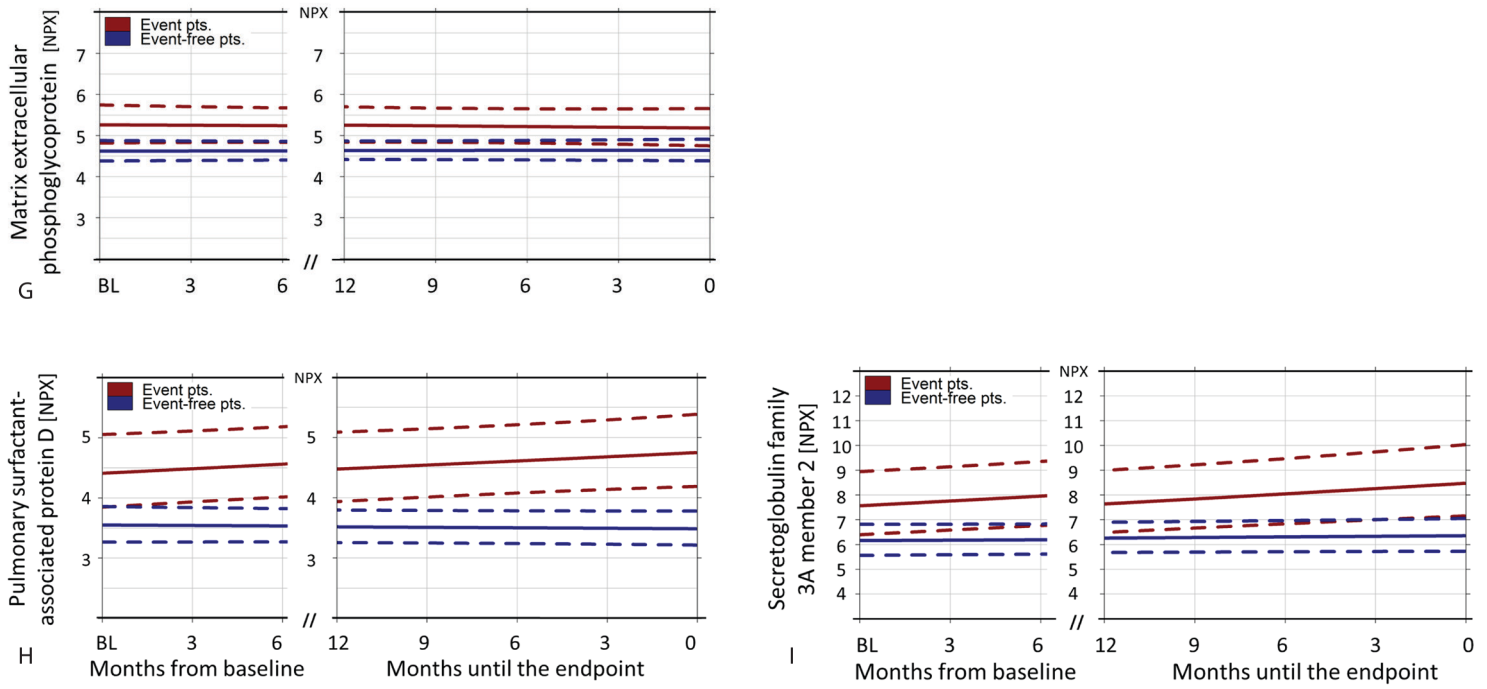

FIGURE 2 Average temporal evolution of candidate biomarkers during follow-up. Average evolution in patients who reached the composite endpoint (solid red line), and in endpoint-free patients (solid blue line). Dashed lines represent the 95% confidence interval. X-axis depicts the time from baseline (left part of the x-axis), and time remaining to the event (patients who experienced incident events) or last sample moment (patients who remained event-free) (right part of the x-axis). Biomarker levels are presented on the y-axis. **A.** Osteopontin (OPN), **B.** Osteoprotegerin (OPG), **C.** Epidermal growth factor receptor (EGFR), **D**. Heparin-binding protein (HBP), **E.** Trefoil factor-3 (TFF-3), **F.** Kallikrein-6 (KLK-6) **G.** Matrix extracellular phosphoglycoprotein (MEPE). **H.** Pulmonary surfactant-associated protein-D (PSP-D) **I.** Secretoglobulin family 3A-member-2 (SCGB3A2).

TABLE 3 **Associations between candidate biomarkers and the composite endpoint.**

	Crude model		Clinical data		Clinical data & time-varying medication		Time-varying cardiac biomarkers & HF-type	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Levels (per 1SD increase)								
Cardiorenal biomarkers								
OPN	2.31 (1.76–3.15)	<0.001*	2.45 (1.64–3.68)	<0.001*	2.78 (2.03–3.80)	<0.001*	1.64 (1.16–2.32)	0.006*
OPG	2.23 (1.69–3.00)	<0.001*	2.76 (1.90–4.16)	<0.001*	2.31 (1.72–3.10)	<0.001*	1.68 (1.21–2.32)	0.002*
EGFR	0.77 (0.54–1.08)	0.13	x		xx		x	
HBP	1.36 (1.09-1.70)	0.006*	1.49 (1.15–1.88)	0.002*	1.65 (1.32–2.06)	<0.001*	1.60 (1.24–2.05)	<0.001*
TFF3	2.20 (1.75–2.82)	<0.001*	2.38 (1.73–3.33)	<0.001*	2.35 (1.84–2.99)	<0.001*	1.29 (0.94–1.77)	0.11
KLK-6	1.60 (1.25–2.04)	<0.001*	1.45 (1.07–1.94)	0.014	1.61 (1.17–2.22)	0.003*	0.95 (0.70–1.29)	0.74
MEPE	1.35 (1.04–1.75)	0.02	1.06 (0.79–1.42)	0.66	xx		0.76 (0.57–1.01)	0.05
Pulmonary biomarkers								
PSP-D	1.66 (1.28–2.12)	<0.001*	1.51 (1.15–1.95)	0.002*	1.63 (1.23–2.16)	0.001*	1.16 (0.89–1.50)	0.26
SCGB3A2	1.44 (1.17–1.77)	<0.001*	1.32 (1.02–1.69)	0.032	1.37 (1.08–1.73)	0.008	1.06 (0.81–1.40)	0.67
Slope (per 0.1SD increase/year)[a]								
Cardiorenal biomarkers								
OPN	1.14 (1.03–1.29)	0.010	1.12 (1.00–1.29)	0.046	1.08 (1.03–1.14)	0.003*	1.05 (1.00–1.12)	0.07
OPG	1.24 (1.14–1.38)	<0.001*	1.48 (1.19–1.88)	0.004*	1.15 (1.08–1.23)	<0.001*	1.09 (1.03–1.16)	0.003*
EGFR	x		x		xx		x	
HBP	0.87 (0.77–1.08)	0.19	0.92 (0.73–0.19)	0.62	xx		1.03 (0.99–1.07)	0.13
TFF3	1.31 (1.17–1.49)	<0.001*	1.55 (1.30–1.87)	<0.001*	1.19 (1.11–1.28)	<0.001*	1.15 (1.07–1.23)	<0.001*

continued

	Crude model		Clinical data		Clinical data & time-varying medication		Time-varying cardiac biomarkers & HF-type	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
KLK-6	1.01 (0.76–1.38)	0.99	1.05 (0.66–1.77)	0.90	xx		1.02 (0.95–1.10)	0.58
MEPE	0.96 (0.86–1.10)	0.57	1.03 (0.91–1.16)	0.66	xx		1.02 (0.95–1.09)	0.61
Pulmonary biomarkers								
PSP-D	1.32 (1.21–1.47)	<0.001*	1.52 (1.32–1.78)	<0.001*	1.12 (1.04–1.19)	0.001*	1.10 (1.04–1.16)	<0.001*
SCGB3A2	1.33 (1.18–1.53)	<0.001*	1.39 (1.19–1.67)	<0.001*	1.10 (1.102–1.20)	0.020	1.08 (1.00–1.17)	0.05

OPN, osteopontin; OPG, osteoprotegerin; EGFR, epidermal growth factor receptor; HBP, heparin-binding protein; TFF3, trefoil factor 3; PSP-D, pulmonary surfactant-associated protein D; SCGB3A2, secretoglobulin family 3A member 2; KLK-6, kallikrein-6; MEPE, matrix extracellular phosphoglycoprotein.

Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 1SD increase of the level and per 0.1SD increase of the annual slope at any point in time during follow-up. **Crude model**: Cox model unadjusted, LME model adjusted for sampling time. **Clinical model**: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, an eGFR, and sampling time (LME); **Clinical & time-varying medication model:** time-dependent Cox model using marker's fitted values from clinical model adjusted for total daily doses of equivalents of carvedilol, enalapril, furosemide, and spironolactone during follow-up. **Time-varying cardiac biomarkers & HF-type model**: time-dependent Cox model using marker's fitted values adjusted for type of HF (HFrEF vs. HFpEF) and time-varying NT-proBNP and cardiac troponin T collected at the same time points during follow-up as the biomarker of interest. Cox and LME models adjusted for baseline NT-proBNP and hs-cTnT, and sampling time (LME).

[a] Annual slopes were additionally adjusted for the levels of repeatedly measured marker during follow-up;

* p-value below the corrected significance level for multiple testing (p<0.007);

x, not performed because repeatedly measured level was not significant;

xx, not performed because marker's levels/slope was not significant in the clinical model.

After adjustment for clinical characteristics and repeatedly assessed CHF pharmacological treatment, OPN, OPG, HBP, TFF3, KLK-6, and PSP-D independently predicted the endpoint (per 1SD increase of marker levels: hazard ratio [95%CI] 2.78 [2.03–3.08], 2.31 [1.72–3.10], 1.65 [1.32–2.06], 2.35 [1.84–2.99], 1.61 [1.17–2.22], 1.12 [1.04–1.19], each $p<0.008$). Levels of these biomarkers, except for KLK-6 and PSP-D, remained significant predictors after adjustment for time-varying levels of two established cardiac biomarkers (NT-proBNP and hs-cTnT). Independently of their absolute levels, the slopes of OPG, TFF3, and PSP-D remained robust clinical predictors after adjusting for clinical characteristics and repeatedly assessed CHF pharmacological treatment and cardiac biomarkers (Table 3).

DISCUSSION

This study is the first to demonstrate that temporal trends in levels of OPN, OPG, and HBP strongly predict clinical outcome in CHF. Moreover, independent of the absolute level of the biomarker, higher slopes of OPG, TFF-3, and PSP-D trajectories were also strong clinical predictors. Importantly, all associations with adverse outcomes were independent of patients' clinical profiles, CHF pharmacological treatment and known cardiac biomarkers measured repeatedly during follow-up. Therefore, these candidate biomarkers may become relevant for clinical practice as they might further define a patient's risk, but also for future HF trials as they might help design more effective biomarker-guided therapy.

Recently, we have demonstrated in the same cohort that temporal patterns of NT-proBNP, troponin T and C-reactive protein are associated with adverse outcome.[23] Our current investigation extends these findings to several novel cardio-renal and pulmonary candidate biomarkers. OPN was previously found to be significantly increased in critically ill patients with AKI compared to those without AKI.[27] Moreover, both animal and human studies have shown that OPN is upregulated in left ventricular hypertrophy, diabetic and dilated cardiomyopathy.[39-42] Interestingly, a small-scale study of CHF patients undergoing cardiac resynchronization therapy (CRT) showed that CRT-responders had significantly lower circulating OPN levels than non-responders.[43] Thus, it is apparent that OPN is involved both in cardiac and renal damage. However, up till now, there have been insufficient data to address the temporal relationship of OPN with adverse clinical outcomes. To this end, our results demonstrate that repeatedly measured OPN levels, but not the slope, are clinically relevant for risk stratification of CHF patients. Taken together, the re-assessment of OPN levels might not only help to update a patient's risk estimates, but may also serve as a potential response-indicator to HF therapy, However, the latter

application of OPN levels warrants confirmation in subsequent clinical studies.

OPG levels predicted progression of vascular calcification and survival in pre-dialysis, dialysis, and renal-transplant patients.[11] In CKD patients, OPG levels were found to be markedly increased in those who had diabetes, which was also observed in our CHF patients.[28] In patients with post-infarction or chronic HF, OPG levels predicted death after acute coronary syndrome and HF-hospitalizations.[29,30,44] However, it is here that our study extends existing evidence by showing that OPG levels dynamically increase as the adverse event such as HF-hospitalization or death approaches. Importantly, the patient's risk entailed by this temporal increase (i.e., higher slope of the OPG trajectory) was independent of OPG levels. In other words, in two patients who have the same "high" OPG levels, it is important whether the OPG levels were high but steady (zero slope) or were increasing prior to assessment (increasing slope). In the latter case, our study shows that every 0.1SD increase in the slope will translate into a 24% higher risk of the event. This information may be used to additionally refine the patients' risk assessment. Interestingly, we also found that patients who were on higher doses of renin-angiotensin-system (RAS) blockers had lower OPG levels. This is indirectly supported by Tsuruda et al. who demonstrated that OPG levels increase in response to cardiac damage during angiotensin II-induced hypertrophy in mice.[45] Therefore, the question is raised whether serial assessment of circulating OPG may be used to identify patients who respond poorly to RAS inhibition. In case OPG does not decrease after RAS inhibition, therapy might be intensified in order to prevent pathological cardiac remodeling.

TFF-3 was found to be upregulated after ischemic myocardial injury in mice.[46] The same authors showed that administration of TFF-3 significantly reduced the infarct size suggesting a cardioprotective effect. In CKD, TFF-3 was found to predict onset of CKD and poor survival.[31] However, data on the prognostic role of TFF-3 in CHF is currently lacking. Hence, this study is the first to demonstrate that increasing slope of the TFF-3 trajectory is a strong clinical predictor in CHF. The importance of TFF-3 in the pathophysiology of CHF is also supported by the network analysis that showed that TFF-3 was the hub within the currently investigated biomarker network. Still, the exact mechanisms of the actions of TFF-3 and its potential use for targeting HF therapy remain to be investigated.

In critically ill patients, HBP was found to be associated with respiratory and circulatory failure, infection-related organ dysfunction, and mortality.[47,48] However, to our best knowledge, there is no previous publication on the role of HBP in CHF. Our study provides strong evidence that HBP is also implicated in CHF by showing a significant association with cardiac decompensation and mortality. Al-

though HBP was independently associated with eGFR, it is unclear whether renal dysfunction is the only factor that contributes to the pool of circulating HBP in CHF. Nevertheless, this study establishes a basis for further investigations on the role of HBP in CHF.

Finally, the pulmonary biomarkers were increased and associated with the primary endpoint independently of the patients' clinical profiles and pharmacological treatment during follow-up. However, only higher slope of PSP-D remained significant predictor after adjustment for time-varying cardiac biomarkers. The fact that the current study population was in a relatively good condition (74% was in NYHA class I-II) may have contributed to the inability to demonstrate robust associations, as lung damage may be expected to manifest itself prominently only with more advanced stages of CHF.[3] Taken together, PSP-D and SCGB3A2 are promising markers and warrant further exploration in more severe stages of CHF.

We found that the new candidate biomarkers studied here are related to the patients' clinical characteristics. Limited data are available on this topic in patients with CHF. Secondly, in this study we utilized a network analysis which may help us to further specify the role of emerging biomarkers in heart failure by analyzing their inter-biomarker relations. In this regard, OPN and TFF-3 were identified as the hubs within the current network, and these findings were subsequently strengthened by the fact that these biomarkers also carried the highest crude risk of adverse events. Thirdly, this study is unique in showing that not only the levels, but also the slopes of biomarker trajectories (i.e., information on how much a marker was increasing, decreasing, or was stable in approach to a subsequent adverse cardiac event) are relevant for risk assessment. As such, temporal biomarker profiles may potentially help to identify the patients who respond poorly to treatment. This may enable timely adaptation of therapy, thereby preventing future events to occur. Finally, our results indicate a promising role of these new biomarkers in defining more effective biomarker-guided therapy, rather than the current approach where therapy is largely based on symptoms and ejection fraction.[49]

Study limitations

Firstly, this cohort consisted mainly of HFrEF patients. The low number of HFpEF patients is most likely attributable to the fact that in the Netherlands, most HFpEF patients are followed in secondary referral centres or by the general practitioner, while the current study was performed in two tertiary referral centres. Potential inclusion bias is not a likely reason for the low HpEF rate, because all consecutive patients were screened in both participating centres. Secondly, enrolled CHF patients were in a

better health condition than previously reported CHF populations. Yet, we were able to demonstrate, even in this 'less sick' CHF population, that several biomarkers are strongly associated with the clinical outcomes. Third, re-hospitalization for HF represented the majority of the composite endpoint. Investigation of individual, 'harder' endpoints such as cardiovascular mortality is advisable, but warrants larger numbers of such endpoints. Finally, future research should focus on better standardization of the assays and reproducibility in other CHF cohorts in order to successfully translate these emerging biomarkers into daily clinical practice.

CONCLUSION

Repeatedly-measured levels of OPN, OPG, and HBP, and slopes of OPG, TFF-3, and PSP-D strongly predict clinical outcome during outpatient follow-up in CHF. The use of these candidate markers may be clinically relevant as they may further refine a patient's risk assessment and provide additional pathophysiological insights into CHF.

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SUPPLEMENTARY INFORMATION

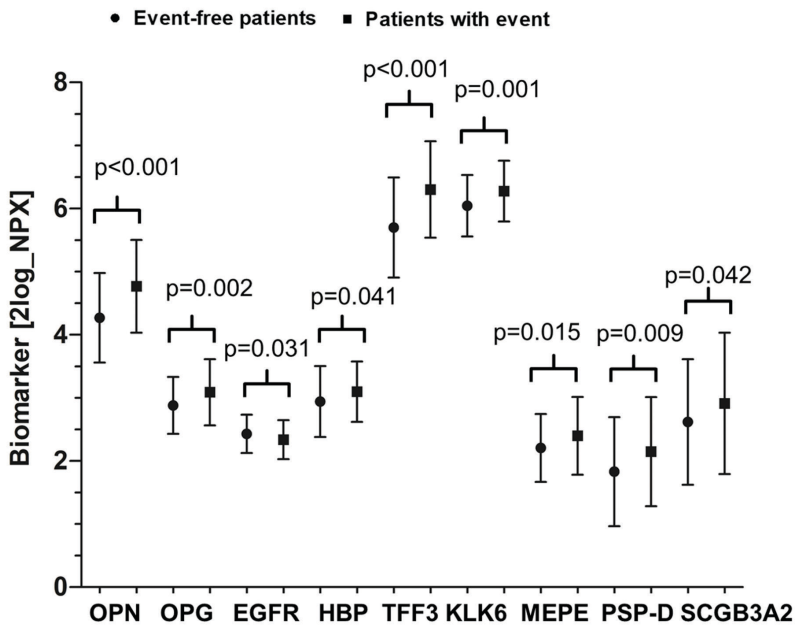

FIGURE S1 Baseline levels of candidate biomarkers in relation to the occurrence of the composite endpoint. T-test was applied to test the differences in baseline levels between the patients who later reached the composite endpoint and those who did not. OPN, osteopontin; OPG, osteoprotegerin; EGFR, epidermal growth factor receptor; HBP, heparin-binding protein; TFF3, trefoil factor 3; PSP-D, pulmonary surfactant-associated protein D; SCGB3A2, secretoglobulin family 3A member 2; KLK-6, kallikrein-6; MEPE, matrix extracellular phosphoglycoprotein.

TABLE S1 Association between time-varying HF medication doses and candidate biomarkers during follow-up.

Time-varying dependent variable	Time-varying independent variable							
	Carvedilol eqv. per 50 mg		Enalapril eqv. per 40 mg		Furosemide eqv. per 40 mg		Spironolactone eqv. per 25 mg	
	β (95%CI)	p-value	β (95%CI)	p-value	β (95%CI)	p-value	β (95%CI)	p-value
OPN	0.03 (-0.08 to 0.15)	0.57	-0.27 (-0.45 to -0.10)	0.003	0.07 (0.04 to 0.10)	<0.001	-0.07 (-0.23 to 0.09)	0.39
OPG	-0.05 (-0.17 to 0.07)	0.43	-0.26 (-0.44 to -0.08)	0.005	0.03 (-0.00 to 0.06)	0.07	-0.20 (-0.36 to -0.04)	0.015
EGFR	-0.04 (-0.16 to 0.09)	0.56	0.18 (0.00 to 0.35)	0.05	0.01 (-0.01 to 0.04)	0.33	-0.15 (-0.31 to 0.02)	0.08
HBP	-0.05 (-0.16 to 0.06)	0.40	-0.05 (-0.21 to 0.12)	0.56	0.03 (0.00 to 0.07)	0.05	-0.13 (-0.29 to 0.02)	0.08
TFF3	-0.01 (-0.11 to 0.08)	0.78	-0.11 (-0.26 to 0.05)	0.18	0.05 (0.02 to 0.08)	<0.001	0.02 (-0.12 to 0.15)	0.82
KLK-6	-0.01 (-0.12 to 0.11)	0.89	-0.12 (-0.30 to 0.05)	0.15	0.04 (0.01 to 0.07)	0.011	-0.28 (-0.44 to -0.13)	<0.001
MEPE	0.07 (-0.05 to 0.19)	0.27	0.02 (-0.16 to 0.20)	0.81	0.05 (0.02 to 0.08)	0.001	-0.04 (-0.20 to 0.13)	0.66
PSP-D	0.00 (-0.10 to 0.10)	0.99	-0.21 (-0.38 to -0.05)	0.013	0.01 (-0.02 to 0.03)	0.50	-0.22 (-0.37 to -0.07)	0.004
SCGB3A2	0.02 (-0.09 to 0.11)	0.81	-0.23 (-0.38 to -0.07)	0.004	0.02 (-0.01 to 0.04)	0.15	-0.14 (-0.27 to -0.01)	0.04

OPN, osteopontin; OPG, osteoprotegerin; EGFR, epidermal growth factor receptor; HBP, heparin-binding protein; TFF3, trefoil factor 3; PSP-D, pulmonary surfactant-associated protein D; SCGB3A2, secretoglobulin family 3A member 2; KLK-6, kallikrein-6; MEPE, matrix extracellular phosphoglycoprotein; eqv. equivalent total daily dose of medication. Linear mixed-effects (LME) models were applied to estimate the temporal effects of HF medication doses on biomarker evolution over time. The models were adjusted for sampling time in the fixed- and random effects part, and intercept and slope were included in the random-effects design matrix. The effects of time-varying HF medication doses are given as β (95% confidence interval) for 1SD differences of biomarkers as measured on the 2log scale. For example, if a furosemide equivalent dose would increase for 40 mg, OPN levels would increase for 0,07SD on the 2log scale.

CHAPTER 7

Cardiometabolic Biomarkers and their Temporal Patterns Predict Poor Outcome in Chronic Heart Failure The Bio-SHiFT study

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ABSTRACT

Background

Multiple hormonal and metabolic alterations occur in chronic heart failure (CHF), but their proper monitoring during clinically silent progression of CHF remains challenging. Hence, our objective was to explore whether temporal patterns of six emerging cardiometabolic biomarkers predict future adverse clinical events in stable patients with CHF.

Methods

In 263 CHF patients, we determined the risk of a composite endpoint of HF-hospitalization, cardiac death, LVAD-implantation and heart transplantation in relation to serially assessed blood biomarker levels and slopes (i.e., rate of biomarker change per year). During 2.2 years of follow-up, we repeatedly measured insulin-like growth binding protein 1, 2, and 7 (IGFBP-1, IGFBP-2, IGFBP-7), adipose fatty acid-binding protein 4 (FABP-4), resistin, and chemerin (567 samples in total).

Results

Serially measured IGFBP-1, IGFBP-2, IGFBP-7, and FABP-4 levels predicted the endpoint (univariable HR [95% confidence interval] per 1SD increase: 3.34 [2.43–4.87], 2.86 [2.10–3.92], 2.45 [1.91–3.13], and 2.46 [1.88–3.24], respectively). Independently of the biomarkers' levels, their slopes were also strong clinical predictors (per 0.1SD increase/year: 1.20 [1.11–1.31], 1.27 [1.14–1.45], 1.23 [1.11–1.37], and 1.27 [1.12–1.48]). All associations persisted after multivariable adjustment for patient baseline characteristics, baseline NT-proBNP and cardiac troponin T, and pharmacological treatment during follow-up.

Conclusions

The temporal patterns of IGFBP-1, IGFBP-2, IGFBP-7, and adipose FABP-4 predict adverse clinical outcomes during outpatient follow-up of CHF patients, and may be clinically relevant as they could help detect more aggressive CHF forms and assess patient prognosis, and ultimately aid in designing more effective biomarker-guided therapy.

INTRODUCTION

Chronic heart failure (CHF) is a clinical syndrome characterized by recurrent episodes of decompensation that require constant therapeutic interventions.[1] After occurrence of initial cardiac alterations in heart failure, the failing heart also induces abnormalities in peripheral organs including the lungs, liver, kidneys, gastrointestinal tract, skeletal muscles, and endocrine system.[2] Together, these abnormalities cause the overall energy balance to shift towards a catabolic state, leading to exercise intolerance and weight loss, both of which strongly determine poor outcome.[3,4] In this context, circulating biomarkers could be an effective clinical tool, as these cellular signals naturally precede the patient's functional decline, and may therefore provide early tissue-specific information on CHF. Similarly, their temporal patterns could help in monitoring disease progression even in the presymptomatic phase, potentially enabling physicians to timely modify therapy to prevent impending decompensation.

Although it has long been known that multiple hormonal and metabolic alterations occur in CHF[5], the biomarkers that reflect these alterations have only recently received increasing attention with the upcoming use of modern -omics technologies that allow us to discover new highly sensitive proteins.[6] To date, ongoing controversy exists concerning the role of these cardiometabolic biomarkers in CHF. Studies have suggested that insulin-like growth factor binding proteins (IGFBPs) 1, 2, and 7 are increased in CHF and are associated with adverse outcomes after myocardial infraction.[7-9] IGFBPs regulate insulin-like growth factor (IGF) activity which is crucial for indirect effects of growth hormone (GH).[10] Of note is that IGFBPs also exhibit IGF-independent effects on the cardiovascular system.[11] In this way, the IGF-IGFBPs system has an important role in the regulation of cardiac remodeling, myocardial contractility, and vascular system function.[10] Similarly, the adipose tissue acts as an endocrine organ by secreting adipokines which are involved in a plethora of metabolic functions including glucose and lipid metabolism, inflammation, atherosclerosis, and cardiac remodeling.[12] Among secreted adipokines, fatty acid-binding protein (FABP)-4, resistin, and chemerin have recently been linked to CHF.[13-15] Nevertheless, the scientific evidence on these biomarkers in CHF is limited, and their potential utility remains undetermined.

Therefore, we investigated the associations of the temporal patterns of cardiometabolic biomarker levels and biomarker slopes (i.e., rates of biomarker change per year) with adverse clinical events in CHF patients who had undergone 3-monthly repeated blood sampling during their outpatient follow-up.

METHODS

CHF cohort

The Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT) is a prospective cohort study of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Noordwest Ziekenhuisgroep, Alkmaar, the Netherlands.[16] Patients were included if aged ≥18 years, capable of understanding and signing informed consent, and if CHF had been diagnosed ≥3 months ago according to European Society of Cardiology guidelines.[1,17,18] Patients were ambulatory and stable, i.e., they had not been hospitalized for HF in the past three months. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients. This investigation comprised 263 CHF patients enrolled during the first inclusion round period (October 2011 until June 2013).

Baseline assessment

All patients were evaluated by research physicians, who collected information on HF-related symptoms, NYHA class, and performed a physical examination. Information on HF etiology, left ventricular ejection fraction, cardiovascular risk factors, medical history and treatment was retrieved primarily from hospital records and was checked in case of ambiguities. History of cardiovascular and other comorbidities was defined as their clinical diagnosis as recorded in the medical file.

Follow-up and study endpoints

During the study, all patients were routinely followed at the outpatient clinic by treating physicians who were blinded for biomarker sampling. Additionally, study follow-up visits were predefined and scheduled every 3 months (±1 month). This 3-month interval was chosen to ensure that blood sampling occurred as often as possible during a relatively long follow-up period (>2 years), while keeping the study burden acceptable for this CHF population. At each study follow-up visit, a short medical evaluation was performed and samples were collected. During follow-up, all medication changes and occurrence of hospitalizations for HF, MI, PCI, CABG, arrhythmias, and CVA, cardiac transplantation, left ventricular assist device (LVAD) implantation and mortality, were recorded in the electronic case

report forms, and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

The composite endpoint comprised cardiac death, cardiac transplantation, LVAD implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalization for acute or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with two of the following: BNP or NT-proBNP >3x upper limit of normal, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.[1]

Study measurements and laboratory analysis

Blood samples were collected at baseline and at each study follow-up visit, and were processed and stored at -80°C within two hours after collection. Treating physicians were unaware of biomarker results as biomarkers were measured batch-wise after completion of follow-up. Thus, the biomarker measurements did not lead to drug adjustments. All patients received usual care. All laboratory personnel was blinded for clinical data and patient outcomes.

For efficiency, for the current investigation we selected all baseline samples, the two samples closest in time to the primary composite endpoint, and the last sample available for patients in whom the primary endpoint did not occur during follow-up.

Glomerular filtration rate (GFR) was determined by the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation validated in HF patients.[19]

The Olink multiplex PEA platform for new biomarkers

The Cardiovascular (CVD) panel III (Olink Proteomics AB, Uppsala, Sweden) was used for analysis of high-abundance proteins. The proteins present in this Olink panel were selected because either they have a proven pathophysiological role in cardiovascular disease, or because they are promising in this respect but yet unexplored. This assay is based on PEA (proximity extension assay) technology.[6] In brief, the assay uses two oligonucleotide-labeled antibodies to bind to their respec-

tive target proteins in the sample. When the two antibodies are in close proximity, a new PCR target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using standard real-time PCR. Each sample includes two incubations, one extension, and one detection control to determine the lower limit of detection and normalize the measurements. The biomarkers are presented in normalized protein expression (NPX) units on a 2log scale. In a validation study, the mean intra-assay and inter-assay coefficients of variation were 8% and 12%, respectively.[6] For the current investigation, six emerging cardiometabolic biomarkers (IGFBP-1, IGFBP-2, IGFBP-7, FABP-4, resistin, and chemerin) were examined.

Statistical analysis

For the analysis, we used the Z-score (i.e., the standardized form) of the 2log-transformed biomarkers to allow for direct comparisons of different biomarkers. For the network analysis we used only the biomarkers that showed significant correlations based on Pearson's correlation coefficients ($p<0.05$). We assessed the clustering coefficient as a measure of the degree to which biomarkers tend to cluster together, where higher coefficients suggest a certain centrality of a biomarker within the network.[20]

To study the effect of baseline characteristics on repeatedly measured biomarkers, linear mixed-effects (LME) models were performed using biomarkers as the dependent variables and baseline characteristics as the independent variables (fixed part). The sampling time was entered into the fixed- and random parts of the models.

To estimate the associations between patient-specific biomarker levels and survival, we applied a joint modeling (JM) analysis that combines LME models for repeated measurements, and Cox survival analysis for time-to-event data.[21] For both the fixed- and random-effects parts of the LME models, linear terms were used for sampling times, and both intercepts and slopes were included in the random-effects design matrix. This allowed the markers' trajectories to differ at baseline and over time. We also estimated the time-dependent slope (i.e., rate of change) of each biomarker from these joint models, indicating whether and by how much the levels are increasing or decreasing and how they relate to patient prognosis. The slope mathematically corresponds to the first derivative of a marker's trajectory, and is presented as an annual change in Z-scores (i.e., delta Z-scores per year).

Besides sampling time, all markers were adjusted as follows: (1) clinical model: Cox and LME models were adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and eGFR; (2) clinical & time-varying HF medication model: after adjusting for clinical characteristics, biomarker values were extracted from the joint models and entered simultaneously with equivalent doses of carvedilol, enalapril, furosemide, and spironolactone (repeatedly assessed during follow-up) into a time-dependent Cox analysis to examine the incremental value of the new biomarkers over clinical characteristics and medication during follow-up; (3) cardiac biomarker model: Cox and LME models were adjusted for biomarkers of myocardial stretch and damage (NT-proBNP and c-TnT). Data on all variables were complete, except for systolic blood pressure which was missing in <5% of patients and for which imputations were applied using the patients' clinical and outcome data. Results are given as hazard ratios (HR) and 95% confidence intervals (CI) per 1SD increase of the marker's level and per 0.1SD increase of the slope at any time-point during follow-up.

To correct for multiple testing, we performed matrix spectral decomposition which has previously been demonstrated to be more effective than Bonferroni correction.[22] Consequently, the corrected significance level was set at $p < 0.0127$ (0.05/4).

All analyses were performed with R Statistical Software using packages nlme and JMbayes.[21] The network analysis was performed using Gephi software (https://gephi.org) and the matSpD application (https://gump.qimr.edu.au/general/daleN/matSpD) available online.

RESULTS

Baseline characteristics

Table 1 displays the patients' baseline characteristics. Specifically, the patients who reached the composite endpoint were older, more frequently had diabetes, atrial fibrillation, lower systolic blood pressure, higher NYHA class, higher levels of NT-proBNP and cardiac troponin T, and were more frequently on loop diuretics.

TABLE 1 Baseline characteristics in relation to the occurrence of the composite endpoint.

Variable	Total	Composite endpoint		p-value
		Yes	No	
n (%)	263 (100)	70 (27)	193 (73)	
Demographics				
Age, years (mean ± SD)	67±13	69±13	66±12	0.05
Men, n (%)	189 (72)	53 (76)	136 (70)	0.41
Clinical characteristics				
BMI, kg/m^2	27.5±4.7	27.6±4.8	27.4±4.7	0.80
Heart rate, b.p.m.	67±12	69±13	67±11	0.31
SBP, mmHg	122±20	117±17	124±21	0.02
DBP, mmHg	72±11	70±10	73±11	0.06
Features of heart failure				
NYHA class III or IV, n (%)	69 (26)	31 (44)	38 (20)	< 0.001
HF-rEF n (%)	250 (95)	66 (94)	184 (95)	0.75
HF-pEF n (%)	13 (5)	4 (6)	9 (5)	
LVEF, %	32±11	30±11	33±10	0.18
NT pro-BNP (pmol/L) †	137.3 (51.7–272.6)	282.4 (176.4–517.4)	95.3 (31.72–207.7)	< 0.001
Hs-TnT (ng/L) †	18.0 (9.5–33.2)	31.9 (20.6–49.7)	13.9 (8.4–26.7)	< 0.001
Etiology of heart failure, n (%)				
Ischemic	117 (44)	36 (51)	81 (42)	0.17
Hypertension	34 (13)	10 (14)	24 (12)	0.70
Valvular disease	12 (5)	5 (7)	7 (4)	0.23
Cardiomyopathy	68 (26)	15 (21)	53 (28)	0.32
Unknown or Others	32 (12)	4 (6)	28 (15)	
Medical history, n (%)				
Prior MI	96 (36)	32 (46)	64 (33)	0.06
Prior PCI	82 (31)	27 (39)	55 (28)	0.12
Prior CABG	43 (16)	13 (19)	30 (15)	0.57
Atrial fibrillation	106 (40)	36 (51)	70 (36)	0.03
Diabetes	81 (31)	32 (46)	49 (25)	0.002
Hypercholesterolemia	96 (36)	30 (43)	66 (34)	0.20
Hypertension	120 (46)	38 (54)	82 (42)	0.09
COPD	31 (12)	12 (17)	19 (10)	0.10
Medication use, n (%)				
Beta-blocker	236 (90)	61 (87)	175 (91)	0.40
ACE-I or ARB	245 (93)	63 (90)	182 (94)	0.22

continued

Variable	Total	Composite endpoint		p-value
		Yes	No	
Diuretics	237 (90)	68 (97)	169 (88)	0.02
Loop diuretics	236 (90)	68 (97)	168 (87)	0.02
Thiazides	7 (3)	3 (4)	4 (2)	0.28
Aldosterone antagonist	179 (68)	53 (76)	126 (65)	0.11
Glomerular function				
Creatinine, mg/dl †	1.18 (0.99–1.49)	1.30(1.02–1.52)	1.17(0.98–1.45)	0.18
eGFR, mL/min/1.73m^2 †	58 (43–76)	53 (40–73)	59 (44–77)	0.16
KDOQI classification, n (%)				0.18
eGFR ≥90	28 (11)	7 (10)	21 (11)	
eGFR 60-89	95 (36)	20 (28)	75 (39)	
eGFR 30-59	119 (45)	37 (53)	82 (42)	
eGFR <30	21 (8)	6 (9)	15 (8)	

BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; NYHA class, New York Heart Association class; HF-rEF, Heart failure with reduced ejection fraction; HF-pEF, heart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction; MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; CVA, cerebrovascular accident; TIA, transitory ischemic attack; COPD, chronic obstructive pulmonary disease; ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; eGFR, estimated glomerular filtration rate.†Median with inter-quartile range (IQR).

Follow-up and study endpoints

During a median of 2.2 (IQR: 1.4–2.5) years of follow-up, we collected a total of 1984 blood samples at fixed 3-month intervals (per patient: 9 [IQR: 5–10] samples), and measured biomarkers in all samples collected at baseline, the two samples closest in time to the composite endpoint, and the last sample available for event-free patients (567 samples in total). During the follow-up, 70 (27%) patients experienced the composite endpoint. Specifically, 56 patients were re-hospitalized for acute or worsened HF, 3 patients underwent heart transplantation, 2 patients underwent LVAD placement, and 9 patients died of cardiovascular causes.

Network analysis

Figure 1 displays baseline inter-marker correlations and crude associations of their serially measured levels with the composite endpoint (for HRs see Table 3). Of note is that all biomarkers correlated with each other and no clustering was present. The strongest correlations were present within the IGFBP biomarker family,

and between these biomarkers and NT-proBNP. Similarly, adipokines correlated strongly with each other.

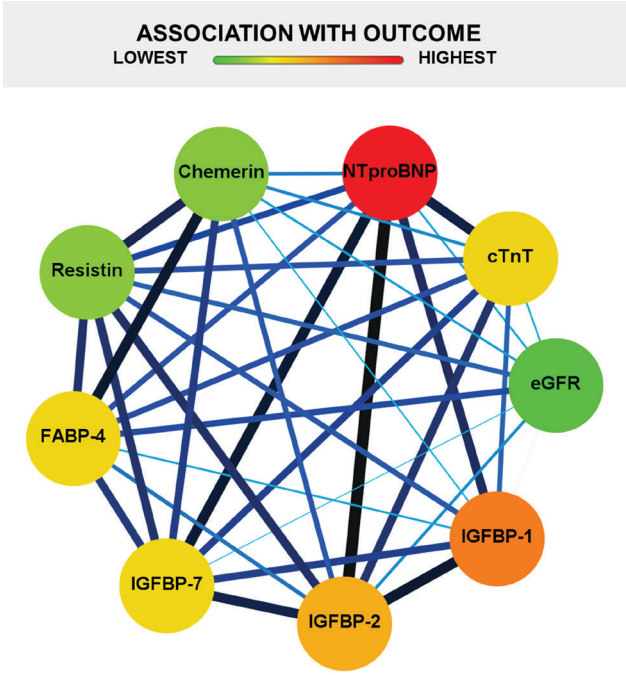


FIGURE 1 Network analysis of cardiometabolic biomarkers depicting inter-marker correlations and associations with the composite endpoint. Node color represents the crude association with the composite endpoint, and ranges from white (the weakest) to black (the strongest). Node size represents the clustering coefficient (a measure of the degree to which biomarkers tend to cluster together suggesting a certain centrality within the biomarker network). The thickness of the line between the biomarkers represents the correlation coefficient (presented only if $p<0.05$); a thicker line represents stronger coefficients.

Patients' clinical characteristics and cardiometabolic biomarkers during follow-up

Table 2 shows the associations between the patients' baseline characteristics and the biomarkers' temporal trends; the reported associations were independent of each other.

IGFBPs

Higher serially measured IGFBP-1 levels during follow-up were associated with increased baseline NT-proBNP and c-TnT, and lower baseline BMI values (per doubling of NT-proBNP: adjusted β [95% confidence interval CI] 0.15SD [0.09; 0.22], p<0.001; c-TnT: 0.13SD [0.02; 0.24], p=0.018; BMI: -0.95SD [-1.38; -0.53], <0.001). Likewise, patients with increased baseline cardiac markers and lower BMI had higher IGFBP-2 levels during follow-up (for regression coefficients see Table 2). The IGFBP-2 levels were also positively associated with older age, higher baseline NYHA class, and impaired baseline eGFR. Similarly, higher IGFBP-7 levels were found in patients with increased baseline cardiac markers and decreased eGFR, and in those who were on higher loop diuretic doses. Patients with atrial fibrillation (AF) also had markedly increased IGFBP-7 levels.

Adipokines

Higher serially measured FABP-4 levels were associated with increased baseline cardiac biomarkers, higher BMI, female sex, and impaired eGFR. Moreover, patients who were on higher baseline β-blockers and loop diuretic doses had higher FABP-4 levels during follow-up. Higher serially measured resistin levels were found in patients with AF, and in those with increased NT-proBNP and decreased eGFR levels. During follow-up, higher serially measured chemerin levels were associated with female gender, higher baseline BMI, decreased eGFR, and higher loop diuretic doses.

Temporal evolutions of IGFBPs and adipokines in relation to study endpoints

Average temporal trajectories of cardiometabolic biomarkers in patients who experienced an incident endpoint and those who did not are displayed in Figure 2. In patients with an incident endpoint, all biomarkers showed increasing patterns to various degrees, with the steepest rise seen in IGFBP-1, IGFBP -2, IGFBP-7, and FABP-4 levels prior to the occurrence of the endpoint.

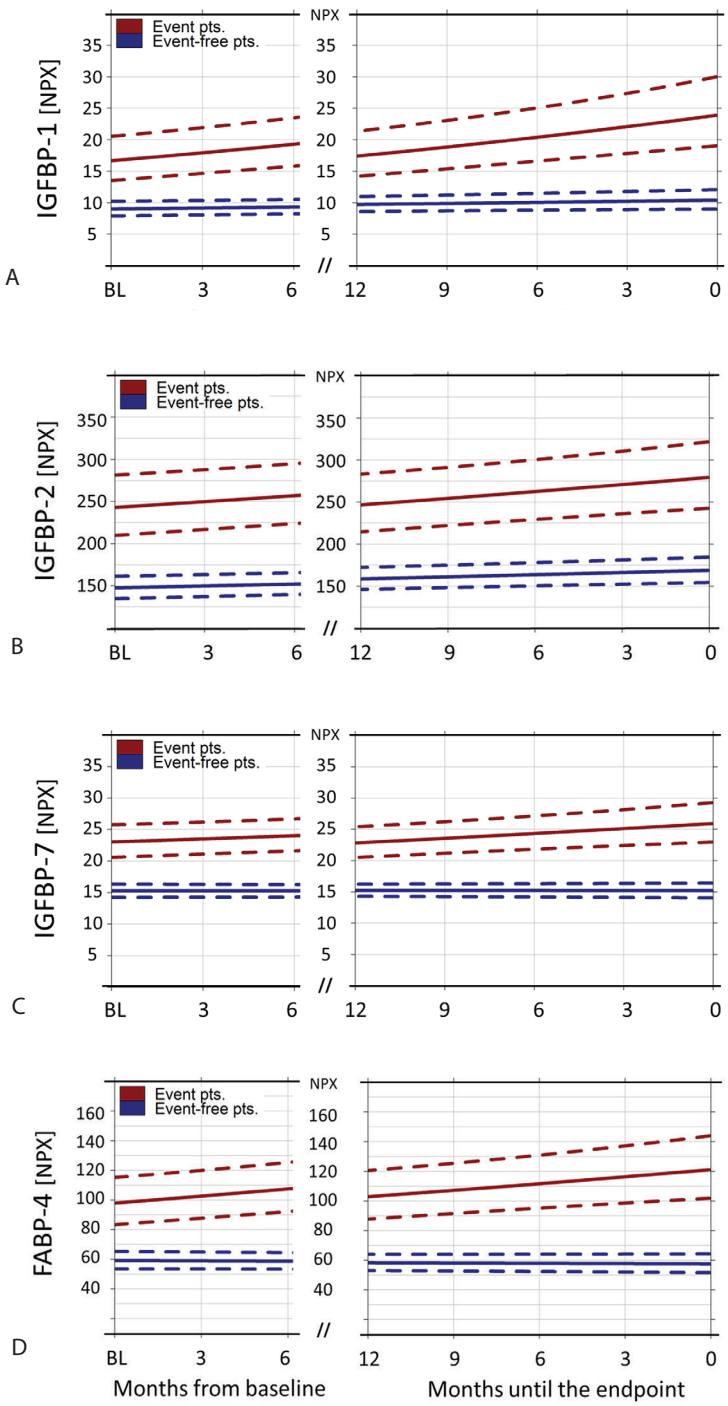
TABLE 2 **Association between baseline characteristics and levels of serially measured biomarkers during follow-up.**

	Dependent variable					
	IGFBP-1		IGFBP-2		IGFBP-7	
Independent variable	**β (95% CI)**	**p-value**	**β (95% CI)**	**p-value**	**β (95% CI)**	**p-value**
Age per 10 yrs.		ns	0.12 (0.01 to 0.20)	0.004		ns
Male sex		ns		ns		ns
BMI per doubling	-0.95 (-1.38 to -0.53)	<0.001	-0.70 (-1.07 to -0.34)	<0.001		ns
NYHA class		ns	0.11 (0.00 to 0.22)	0.05		ns
DM		ns		ns		ns
AF		ns		ns	0.42 (0.23 to 0.60)	<0.001
SBP per 10mmHg		ns		ns		ns
eGFR per 20 ml/min/1.73m^2		ns		ns	-0.10 (-0.19 to -0.01)	0.026
NT-proBNP per doubling	0.15 (0.09 to0.22)	<0.001	0.20 (0.14 to 0.25)	<0.001	0.18 (0.11 to 0.24)	<0.001
cTnT per doubling	0.13 (0.02 to 0.24)	0.018	0.16 (0.07 to 0.25)	<0.001	0.11 (0.00 to 0.21)	0.041
Carvedilol per 50 mg		ns		ns		ns
Enalapril per 40 mg		ns		ns		ns
Furosemide per 40 mg		ns		ns	0.06 (0.02 to 0.11)	0.005
Spironolactone per 25 mg		ns		ns		ns

continued

	Dependent variable					
	FABP-4		Resistin		Chemerin	
Independent variable	**β (95% CI)**	**p-value**	**β (95% CI)**	**p-value**	**β (95% CI)**	**p-value**
Age per 10 yrs.		ns		ns		ns
Male sex	-0.62 (-0.80 to -0.44)	<0.001		ns	-0.38 (-0.60 to -0.16)	<0.001
BMI per doubling	1.41 (1.05 to 1.76)	<0.001		ns	0.51 (0.07 to 0.94)	0.022
NYHA class		ns		ns		ns
DM		ns		ns		ns
AF		ns	0.34 (0.13 to 0.55)	0.001		ns
SBP per 10mmHg		ns		ns		ns
eGFR per 20 ml/min/1.73m^2	-0.23 (-0.31 to -0.15)	<0.001	-0.25 (-0.35 to -0.15)	<0.001	-0.20 (-0.30 to -0.10)	<0.001
NT-proBNP per doubling	0.11 (0.06 to 0.16)	<0.001	0.09 (0.02 to 0.16)	0.011		ns
cTnT per doubling	0.21 (0.12 to 0.30)	<0.001		ns		ns
Carvedilol per 50 mg	0.10 (0.01 to 0.20)	0.038		ns		ns
Enalapril per 40 mg		ns		ns		ns
Furosemide per 40 mg	0.06 (0.03 to 0.10)	0.001		ns	0.08 (0.04 to 0.13)	<0.001
Spironolactone per 25 mg		ns		ns		ns

IGFBP-1, insulin-like growth factor-binding protein 1; IGFBP-2, insulin-like growth factor-binding protein 2; IGFBP-7, insulin-like growth factor-binding protein 7; FABP-4, fatty acid-binding protein 4 (adipocytes); ns, not significant (only the associations with significance level of p-value <0.05 are presented). The effects of the patients' baseline characteristics are given as adjusted β (95% confidence interval) per 1SD differences of biomarkers as measured on the 2log scale. This method allows a direct comparison of the effects on different biomarkers. All βs are adjusted for patients' age, sex, body mass index (BMI), diabetes mellitus (DM), atrial fibrillation (AF), baseline NYHA class, systolic blood pressure (SBP), estimated glomerular filtration rate (eGFR), NT-proBNP levels, cardiac troponin T levels (c-TnT), and equivalent doses of carvedilol, enalapril, furosemide, and spironolactone. Only the associations with significance level of p-value <0.05 are presented.


A
IGFBP-1 [NPX]
Event pts.
Event-free pts.
NPX
35
30
25
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10
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BL
3
6
12
9
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3
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B
IGFBP-2 [NPX]
350
300
250
200
150
C
IGFBP-7 [NPX]
D
FABP-4 [NPX]
160
140
120
100
80
60
40
Months from baseline
Months until the endpoint

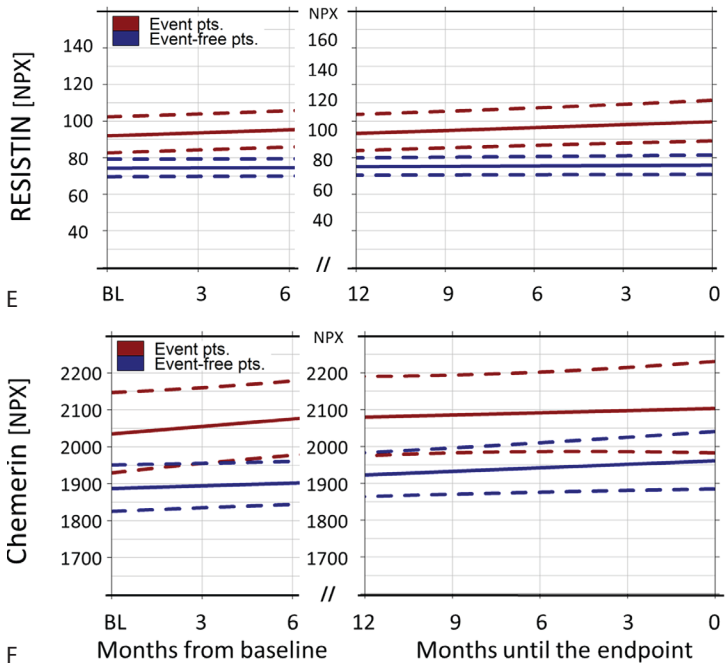

FIGURE 2 Average temporal evolution of IGFBP-1, IGFBP-2, IGFBP-7, FABP-4, resistin, chemerin during follow-up. Average evolution in patients who reached the composite endpoint (solid black line), and in endpoint-free patients (solid gra line). Dashed lines represent the 95% confidence interval. X-axis depicts the time from baseline (BL: left part of the x-axis), and time remaining to the event (patients who experienced incident events) or last sample moment (patients who remained event-free) (right part of the x-axis). Biomarker levels are presented on the y-axis. (a) IGFBP-1 (b) IGFBP-2, (c) IGFBP-7, (d) FABP-4, (e) resistin, (f) chemerin.

Table 3 summarizes the associations of the cardiometabolic biomarkers with the composite endpoint. After adjustment for baseline clinical characteristics and HF pharmacological treatment during follow-up, IGFBP-1, IGFBP-2, IGFBP-7, and FABP-4 independently predicted the endpoint (per 1SD increase of biomarker levels HR [95%CI]: 4.21 [2.96; 6.01], 2.93 [2.11; 4.08], 2.72 [2.06; 3.60], 3.15 [2.36; 4.21], respectively, each $p<0.001$). These biomarkers remained significant predictors even after controlling for baseline NT-proBNP and cTnT levels. Notably, their higher slopes were strong predictors independently of their absolute levels, baseline clinical characteristics and cardiac biomarkers, and HF medication during follow-up. Serially measured resistin predicted the endpoint independently of the patients' clinical characteristics and pharmacological treatment, but lost significance after controlling for cardiac markers. The chemerin did not show clear prognostic value after multivariable adjustments (Table 3).

TABLE 3 **Associations between serially measured cardiometabolic markers and the composite endpoint.**

	Crude model		Clinical model		Clinical & time-varying medication model		Cardiac biomarkers model	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Levels (per 1SD increase)								
IGFBP-1	3.34 (2.43–4.87)	<0.001*	3.10 (2.16–4.70)	<0.001*	4.21 (2.96–6.01)	<0.001*	2.65 (1.78–4.12)	<0.001*
IGFBP-2	2.86 (2.10–3.92)	<0.001*	2.71 (1.87–3.96)	<0.001*	2.93 (2.11–4.08)	<0.001*	2.02 (1.37–2.97)	<0.001*
IGFBP-7	2.45 (1.91–3.13)	<0.001*	2.44 (1.78–3.36)	<0.001*	2.72 (2.06–3.60)	<0.001*	1.96 (1.51–2.62)	<0.001*
FABP-4	2.46 (1.88–3.24)	<0.001*	3.94 (2.61–5.95)	<0.001*	3.15 (2.36–4.21)	<0.001*	1.97 (1.51–2.58)	<0.001*
Resistin	1.75 (1.37–2.21)	<0.001*	1.63 (1.18–2.25)	<0.001*	1.74 (1.34–2.26)	<0.001*	1.27 (0.97–1.68)	0.09
Chemerin	1.72 (1.22–2.50)	<0.001*	1.37 (0.91–2.10)	0.13	xx		1.31 (0.93–1.86)	0.12
Slope (per 0.1SD increase / year)[a]								
IGFBP-1	1.20 (1.11–1.31)	<0.001*	1.50 (1.25–1.86)	<0.001*	1.19 (1.13–1.25)	<0.001*	1.18 (1.10–1.28)	<0.001*
IGFBP-2	1.27 (1.14–1.45)	<0.001*	1.66 (1.27–2.33)	<0.001*	1.13 (1.05–1.22)	0.002*	1.21 (1.10–1.35)	<0.001*
IGFBP-7	1.23 (1.11–1.37)	<0.001*	1.43 (1.16–1.86)	<0.001*	1.18 (1.10–1.25)	<0.001*	1.14 (1.04–1.25)	<0.001*
FABP-4	1.27 (1.12–1.48)	<0.001*	1.57 (1.25–2.04)	<0.001*	1.19 (1.12–1.28)	<0.001*	1.16 (1.05–1.31)	0.002*
Resistin	1.35 (1.21–1.56)	<0.001*	1.51 (1.26–1.90)	<0.001*	1.11 (1.02–1.20)	0.018	1.25 (1.13–1.39)	<0.001*
Chemerin	1.30 (1.10–1.50)	<0.001*	1.67 (1.45–1.96)	<0.001*	1.02 (0.65–1.09)	0.58	1.09 (0.95–1.22)	0.22

Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 1SD increase of the level and per 0.1SD increase of the annual slope of a biomarker at any point in time during follow-up. **Crude model**: Cox model unadjusted, LME model adjusted for sampling time. **Clinical model**: Cox and LME models adjusted for age, sex, body mass index, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, eGFR, and sampling time (LME); **Clinical & time-varying medication model**: time-dependent Cox model using a biomarker's fitted values from clinical model adjusted for total daily doses of equivalents of carvedilol, enalapril, furosemide, and spironolactone during follow-up. **Cardiac biomarkers model**: Cox and LME models adjusted for baseline NT-proBNP and c-TnT, and sampling time (LME).
[a] Annual slopes were additionally adjusted for the levels of serially measured biomarker during follow-up. * p value below the corrected significance level for multiple testing (p<0.0127); xx not performed because marker's levels were not significant in clinical model.

DISCUSSION

This study is the first to demonstrate that temporal patterns of IGFBP-1, IGFBP-2, IGFBP-7, and FABP-4 strongly predict adverse clinical outcomes in CHF. Independently of the absolute biomarker levels, their higher slopes (i.e., higher rates of change) were also strong clinical predictors. All aforementioned associations were robust to the multivariable adjustment for baseline clinical characteristics and cardiac natriuretic peptide and troponin levels, as well as CHF pharmacological treatment during follow-up.

IGFBPs in CHF

In CHF, an impaired anabolic drive with increased GH levels but peripheral GH resistance, and elevated IGFBPs, has been reported.[8,23,24] In this regard, studies have suggested that elevated IGFBPs, especially high-affinity IGF binders such as IGFBP-1 and IGFBP-2, indirectly control anabolic activity via their inhibitory function on the GH–IGF-1 axis.[11] The IGFBPs also directly control cell growth and survival which may also contribute to adverse CHF outcomes.[11] Our study confirms that patients who experienced adverse clinical events had higher levels of all three IGFBPs than event-free patients, which rose during the follow-up. These elevated IGFBPs might also explain the conflicting results of clinical trials on GH therapy in CHF.[25] A meta-analysis on GH treatment showed that CHF patients who had a reduced IGF-1 response to GH administration were less likely to benefit from this treatment. Thus, it may be speculated that elevated IGFBPs may be responsible for the "non-responsiveness" to GH treatment in these patients, but further investigations are needed to confirm this hypothesis.

Besides higher levels, the rise of IGFBP-1, IGFBP-2, and IGFBP-7 over the time-course of CHF strongly predicted adverse outcome. In other words, we found elevated risk in patients in whom the levels were increasing (i.e., a higher slope was present) compared to patients with similar IGFBP levels, but in whom levels remained constant (for details see Figure 3). These new insights into IGFBPs' temporal dynamics is important considering the dynamic nature of myocardial remodeling itself, which has a pivotal role in CHF progression. These findings are novel and carry clinical implications for the monitoring of CHF patients.

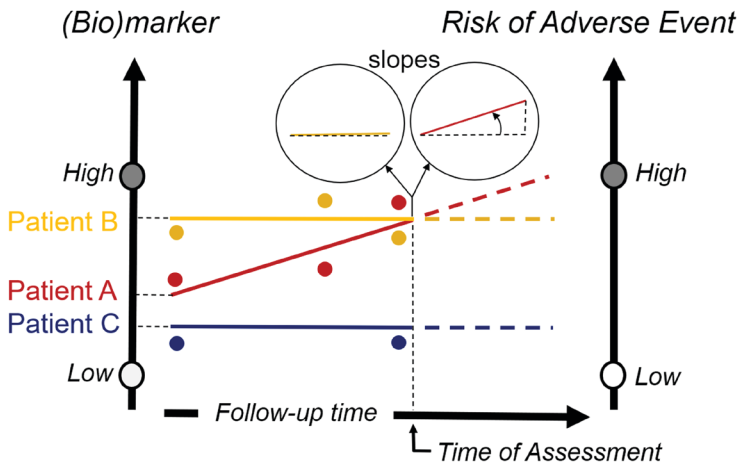

FIGURE 3 An illustration of different aspects of the underlying trajectory of a serially assessed biomarker that may be relevant for a patient's prognosis. Figure illustrates biomarker trajectories of three distinct patients. Their biomarker levels are measured on the y-axis and their follow-up time on the x-axis. At the time of assessment, patient C has lower biomarker levels than patients A and B. Corresponding risk in patient C is lower than in patients A and B. By comparing patients A and B, we observe that they have the same biomarker levels. Thus, if we would like to compare these two patients we have to look at their slopes and relate them to the risk of event. In other words, for two patients with the same biomarker levels, increase in slope provides us with risk estimates related to the adverse event independently of their levels. These risk estimates are provided in Table 3 under *"Slope (per 0.1SD increase / year)"*. The dots represent actual biomarker levels.

All three IGFBPs correlated independently with CHF severity as assessed by cardiac natriuretic peptide and troponin levels. However, IGFBPs differed with respect to their associations with other patient characteristics. Specifically, we found an independent inverse relation between the patients' BMI and IGFBP-1 and IGFBP-2. This has been consistently reported.[7,26] Thus, it may be speculated that the "obesity paradox", in which higher BMI is associated with better survival in CHF[27], might be (partially) mediated through the suppressed adverse effects of IGFBP-1 and IGFBP-2. Yet, this association could not be demonstrated between BMI and IGFBP-7. On its part, IGFBP-7 correlated independently with higher prevalence of atrial fibrillation. In this context, IGFBP-7 has previously been linked to left ventricular (LV) diastolic dysfunction[28] and to increased collagen deposition[29], both of which may contribute to atrial fibrillation. Taking these findings together with the fact that IGFBP-7 belongs to the category of low-affinity IGF binders[30], it appears that direct effects of IGFBP-7 on the myocardium may predominate in the setting of CHF.

Altogether, based on our findings we may tentatively hypothesize that preventing alterations in IGFBPs, by means of lowering IGFBP levels, might potentially help protect the myocardium from further damage.

Adipokines in CHF

Adipose tissue has been identified not only as an energy deposit, but also as a hormonally active organ that releases numerous bioactive molecules called adipokines.[12] Among them, adipose FABP-4 has been linked to metabolism-related cardiac alterations including LV hypertrophy and systolic dysfunction.[31,32] Animal models suggest that adipose FABP-4 causes these cardiac alterations by reducing shortening amplitude and the intracellular systolic Ca^{2+} peak in cardiomyocytes.[33] FABP-4 also predicted the onset of HF among elderly population within the Cardiovascular Health Study.[13] The present study extends these findings by showing that higher FABP-4 levels and higher rate of change in these levels predict adverse clinical events, also in patients with prevalent CHF.

Resistin and chemerin are two other adipokines which are mainly involved in the inflammatory activity underlying cardiovascular diseases.[12] Resistin is largely secreted by mononuclear cells in response to inflammatory stimuli.[34] Animal experiments have demonstrated that in the heart, resistin alters glucose handling, herewith leading to hypertrophy and impaired cardiomyocyte contractility.[35] In murine cardiomyocytes, chemerin was found to induce apoptosis through the activation of several apoptotic mediators.[36] In humans, both resistin and chemerin correlated with LV mass and systolic dysfunction[15,37] but their prognostic role in CHF is less firmly established. In this regard, we found increased levels of both biomarkers in CHF patients who reached adverse events compared to those who did not, but we could not demonstrate their independent prognostic value in CHF.

Cardiometabolic markers as a new link for cardio-renal interaction

We found that repeatedly measured IGFBP-7, FABP-4, resistin, and chemerin are associated with impaired baseline eGFR independently of the patients' characteristics, cardiac markers, and CHF pharmacological treatment. In this context, increased urinary IGFBP-7 has been identified in the settings of acute kidney injury[38], and FABP-4 has been correlated with renal dysfunction and progression of proteinuria.[39] Similarly, resistin and chemerin have been linked to impaired kidney functioning.[14,40] Altogether, this raises the question whether these cardiometabolic

biomarkers also represent another link underlying cardiorenal interaction responsible for worse CHF prognosis. However, additional studies including animal experiments exclusively focusing on this subject are needed to elucidate this promising concept.

Clinical implications

Our findings, together with previous reports, indicate that the use of cardiometabolic biomarkers for monitoring of CHF progression is a rapidly growing area of interest. The current study explores a potential clinical role of these biomarkers to assist in the care of CHF patients through better phenotyping of CHF. We found that CHF patients with higher levels and slopes of IGFBP-1, IGFBP-2, IGFBP-7, and FABP-4 had a more aggressive form of CHF ultimately leading to adverse outcomes such as re-hospitalization or death. This may be important in practice to enable timely adjustment of therapy in patients without clinically overt CHF. These results provide a basis for future studies to further explore this hypothesis. Besides their prognostic role, these biomarkers are bioactive proteins as they activate distinct cell signaling pathways within the IGF-IGFBPs system and adipose-related tissue. Therefore, targeting these biomarkers may be a promising approach in designing more effective biomarker-guided CHF therapy. Finally, future research should focus on better standardization of the assays, and combining the results with genetic analyses may further help to successfully translate these biomarkers into clinical practice.

Study limitations

Firstly, this cohort consisted mainly of HFrEF patients. The low number of HFpEF patients can most likely be attributed to the fact that in the Netherlands, most HFpEF patients are followed in secondary referral centres or by their primary care provider, while our study was performed in two tertiary referral centres. Potential selection bias is not a likely reason for the low rate of HpEF patients, because all consecutive patients were screened in both participating centres. Secondly, although we had trimonthly blood samples available for all patients, because of efficiency reasons only two sampling moments were selected for event-free patients, and three sampling moments for patients with a PE. In previous investigations within this cohort, we have used all available sampling moments to determine NT-proBNP, cTnT, CRP as well as glomerular and tubular renal biomarkers.[16] Those investigations demonstrated that most of these biomarkers show an increase shortly prior to the incident adverse event. Thus, we believe that by selecting baseline

samples, as well as the last two samples prior to the incident endpoint, we retain the most informative measurements while enhancing efficiency.

CONCLUSION

The temporal patterns of IGFBP-1, IGFBP-2, IGFBP-7, and adipose FABP-4 strongly predict adverse clinical outcomes during outpatient follow-up of CHF patients. These biomarkers may, therefore, be relevant for clinical practice as they could help detect more aggressive forms of CHF and assess patient prognosis, and ultimately aid in designing more effective biomarker-guided therapy.

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CHAPTER 8

Temporal Patterns of 14 Blood Biomarkers of Cardiac Remodeling in Relation to Prognosis of Patients with Chronic Heart Failure – The Bio-SHiFT Study

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Submitted

ABSTRACT

Background

Remodeling biomarkers carry high potential for predicting adverse events in chronic heart failure (CHF) patients. However, temporal patterns during the course of CHF, and especially the trajectory prior to an adverse event, are unknown. We studied the prognostic value of temporal patterns of 14 cardiac remodeling biomarker-candidates in stable patients with CHF.

Methods

In 263 CHF patients, we performed trimonthly blood sampling during a median follow-up of 2.2 years. For the analysis, we selected all baseline samples, the two samples closest to the primary endpoint (PE), or the last sample available for endpoint-free patients. Thus, in 567 samples, we measured ST2, Gal-3, Gal-4, GDF-15, MMP-2, 3 and 9, TIMP-4, PLC, AP-N, CASP3, CTSD, CTSZ and CSTB. The PE was a composite of cardiovascular mortality, heart transplantation, left ventricular assist device implantation and HF-hospitalization. Associations between repeatedly-measured biomarker-candidates and the PE were investigated by joint modelling.

Results

Median age was 68 (IQR:59-76) years with 72% men; 70 patients reached the PE. Repeatedly measured ST2, Gal-3, Gal-4, GDF-15, MMP-2 and 9, TIMP-4, PLC, CTSD and CSTB levels were strongly and significantly associated with the PE, and increased as the PE approached. The slopes of biomarker trajectories were also predictors of clinical outcome, independent of their absolute level. Associations persisted after adjustment for clinical characteristics and pharmacological treatment. ST2 was the strongest predictor (HR: 7.55 per SD difference, 95%CI: 5.53-10.30), followed by GDF-15 (4.06, 2.98-5.54) and MMP-2 (3.59, 2.55-5.05).

Conclusions

Temporal patterns of remodeling biomarker-candidates strongly predict adverse clinical outcomes in CHF.

INTRODUCTION

Chronic heart failure (CHF) is a complex syndrome that may result from a diverse spectrum of conditions preventing the left ventricle from properly filling and ejecting blood.[1] Beyond the traditional evaluation of suspected heart failure (HF) patients, the use of biomarkers is on the rise.[2] Circulating blood biomarkers are capable of detecting subtle changes in the pathophysiological processes underlying CHF, and can be measured with relative ease. Not only do they have a crucial role in the diagnosis of HF, but also in risk stratification of patients with CHF.

Since the introduction of natriuretic peptides, interest in other biomarkers has grown exponentially.[3] In this context, biomarkers of cardiac remodeling, which represent complex histological and structural myocardial changes, including cardiac hypertrophy, fibrosis and inflammation4, have recently gained wide attention. Consistent associations have been found between Suppression of tumorigenicity-2 (ST2), Galectin-3 (Gal-3) and Growth differentiation factor 15 (GDF-15) and adverse prognosis in CHF patients.[5-7] Overall, studies performed so far have shown that remodeling biomarkers carry high potential for predicting adverse events in CHF patients.[8]

Since blood biomarkers reflect the disease processes underlying CHF, their levels may be expected to change in accordance with disease severity, as well as prior to adverse events.[9] However, temporal patterns of remodeling biomarkers during the course of CHF, and especially temporal patterns shortly before an adverse event occurs have not yet been investigated in detail. Previous studies have mostly described the value of single, baseline measurements of cardiac remodeling biomarkers for prognosis. Only a few studies have been performed on serial assessment of, for example, ST2[10-12], but these studies were usually relatively small, or re-measured the biomarker during a brief first follow-up period only and then did not re-measure at regular intervals during longer-term follow-up. Furthermore, these studies have mostly used only one repeated measurement and described the change between two measurements, which does not properly capture the underlying temporal trajectory.[13]

Conversely, a recent report from the TRIUMPH study, which performed 7 repeated ST2 measurements during 1-year follow-up, clearly demonstrated the incremental value of temporal patterns derived from such frequent, repeated sampling in patients with acute HF[14], illustrating the need for further research on this topic. Accordingly, the aim of our study was to evaluate temporal patterns of 14 biomarker-candidates of cardiac remodeling and their value for predicting future adverse clinical events in patients with CHF. For this purpose, we performed repeated mea-

surements of the levels of ST2, Gal-3, Galectin-4 (Gal-4), GDF-15, extracellular matrix components, selected proteolytic enzymes and N-terminal pro–B-type natriuretic peptide (NT-proBNP) in 263 stable patients with CHF, and investigated the associations of these biomarker-candidate levels, and changes therein, with adverse clinical events.

METHODS

CHF cohort

The 'Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis' (BioSHiFT) study is a prospective cohort study of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Northwest Clinics, Alkmaar, The Netherlands.[15,16] Patients were included if aged ≥18 years, capable of understanding and signing informed consent, and if CHF had been diagnosed ≥3 months ago according to European Society of Cardiology guidelines.[17,18] Patients were ambulatory and stable, i.e., they had not been hospitalized for HF in the past three months. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients. This investigation comprised 263 CHF patients that were enrolled during the first inclusion period from October 2011 until June 2013. Follow-up lasted until 2015.

Study procedures

All patients were evaluated by research physicians, who collected information on HF-related symptoms, New York Heart Association (NYHA) class, and performed a physical examination. Information on HF etiology, left ventricular ejection fraction, cardiovascular risk factors, medical history and treatment was retrieved primarily from hospital records and was checked in case of ambiguities. History of cardiovascular and other comorbidities was defined as clinical diagnosis thereof reported in the hospital records. Glomerular filtration rate (GFR) was determined by the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation validated in HF patients.[19] Patients were categorized using National Kidney Foundation–Kidney Disease Outcome Quality Initiative (KDOQI) clinical practice guidelines.[20] Baseline NT-proBNP and Cardiac troponin T (hsTnT) were measured in 1 batch in stored serum samples as described before[15], using electrochemiluminescence immunoassays (Elecsys 2010; Roche Diagnostics, Indianapolis, IN).

All patients were followed at the outpatient clinic as part of standard care by their treating physicians, who were blinded for biomarker-candidate results. Additionally, study follow-up visits were predefined and scheduled every 3 months (±1 month). At each study follow-up visit, the research physician performed a short medical evaluation and blood samples were collected. During follow-up, all medication changes and occurrence of hospitalizations for HF, myocardial infarction (MI), percutaneous coronary intervention, coronary artery bypass grafting, arrhythmias, cerebrovascular accident, heart transplantation, left ventricular assist device (LVAD) implantation and mortality, were recorded in the electronic case report forms, and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker-candidate results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

The primary endpoint (PE) was a composite of cardiac death, heart transplantation, LVAD implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. We used the International Classification of Disease-10th revision (ICD-10), from the World Health Organization, to assign the endpoints.[21] Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10 : codes I20-I25), death from other heart disease including HF (codes I30-I45 and I47-I52), sudden cardiac death (code I46), sudden death undefined (code R96) or unwitnessed or ill-described death (codes R98, R99). Hospitalization for acute or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with two of the following: BNP or NT-proBNP >3x upper limit of normal, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.[17]

Laboratory procedures

Blood samples were collected at baseline and at each trimonthly study follow-up visit, and were processed and stored at -80^{o}C within two hours after collection. Treating physicians were unaware of biomarker-candidate results as these biomarker-candidates were measured batchwise after completion of follow-up. Thus, the biomarker-candidate measurements did not lead to drug adjustments. All patients received usual care. All laboratory personnel were blinded for clinical data and patient outcomes.

Selection of blood samples

Blood samples were drawn at each study follow-up visit, which were predefined and scheduled every 3 months (±1 month). Hence, in the first inclusion round of the Bio-SHiFT study which we used for the current investigation, we collected a total of 1984 samples before occurrence of the PE or censoring (9 (5–10) blood samples per patient). For reasons of efficiency, for the current investigation, we made a selection from these 1984 samples: we selected all baseline samples, the last sample available in patients in whom the PE did not occur during follow-up, and the two samples available closest in time prior to the PE (which, by design, were 3 months apart) (Figure 1). Our previous investigations in this cohort have demonstrated that several biomarker-candidates increase in the months prior to the incident adverse event.[15,16] Thus, by selecting the last 2 samples prior to the incident endpoint, we aimed to capture this increase. Conversely, in event-free patients, our previous investigations showed stable biomarker-candidate levels, in which case 1 additional sample suffices. Altogether, our selection amounted to 567 samples for the current analysis.

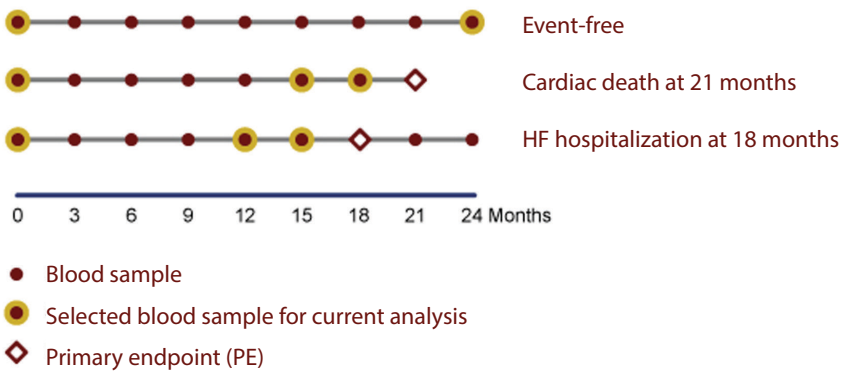


FIGURE 1 Selection of blood samples. At each study follow-up visit, the research physician performed a short medical evaluation and blood samples were collected. Study follow-up visit were predefined and scheduled every 3 months (±1 month). The primary endpoint (PE) was a composite of cardiac death, heart transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. For reasons of efficiency, for the current investigation we selected all baseline samples, the two samples closest in time prior to the PE, and the last sample available in patients in whom the PE did not occur during follow-up. Blood sampling continued after hospitalization, but since hospitalization for the management of acute or worsened HF was considered as PE, the two samples closest in time prior to hospitalization were selected for the current analysis.

Biomarker-candidate measurements

The Cardiovascular (CVD) panel III of the Olink Multiplex platform for new biomarkers (Olink Proteomics AB, Uppsala, Sweden) was used for analysis of high-abundance proteins. The proteins analyzed by the assay were chosen based on their potential to represent aspects of cardiovascular pathophysiology. The assay is based on proximity extension assay technology.[22] In brief, the assay uses two oligonucleotide-labeled antibodies to bind to their respective target proteins in the sample. When the two antibodies are in close proximity, a new polymerase chain reaction (PCR) target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using standard real-time PCR. Four internal controls and two external controls were included in each assay. In a validation study, the mean intra-assay and inter-assay coefficients of variation were 8% and 12%, respectively.[23] The biomarker-candidates are delivered in Normalized Protein Expression (NPX) Units, which are relative units that result from the PCR. They are expressed on a log2 scale where one unit higher NPX value represents a doubling of the measured protein concentrations. This arbitrary unit can thus be used for relative quantification of proteins and comparing the fold changes between groups. For the current investigation, ST2, Gal-3, Gal-4, GDF-15, matrix metalloproteinase (MMP)-2, 3 and 9, Tissue Inhibitor Metalloproteinase (TIMP)-4, Perlecan (PLC), aminopeptidase-N (AP-N), Caspase-3 (CASP3), Cathepsin D (CTSD), Cathepsin Z (CTSZ), Cystatin-B (CSTB) and NT-proBNP were examined.

Statistical analysis

Variables with a normal distribution are presented as mean ± standard deviation (SD), whereas the median and interquartile range (IQR) are presented in case of non-normality. Categorical variables are presented as counts and percentages. Freedom from composite endpoint was assessed using Kaplan-Meier analysis, first for the full cohort and then according to median biomarker-candidate value. Biomarker-candidates as measured by the Olink CVD III panel are presented as arbitrary, relative units (NPX values) on their linear scale (i.e., non-log transformed) in Table 1, Table 2 and Figure 3. In the below mentioned models, we used the Z-score (i.e., the standardized form) of the log2-transformed biomarkers to allow for direct comparisons of different biomarker-candidates.

We applied a joint modeling (JM) analysis to estimate the associations between patient-specific repeated biomarker-candidate levels and the hazard of the PE. JM combines linear mixed effect (LME) models for repeated measurements with Cox

proportional hazard models for the time-to-event data.[24] By doing this, all biomarker-candidate values were corrected for different follow-up durations between patients.[25] We studied the predictive value of absolute biomarker-candidate levels, as well as their rates of change (i.e., the slopes of the longitudinal biomarker trajectories).

In order to adjust for clinical risk determinants and potential confounders, we considered the following pre-defined models: 1) clinical model: LME and Cox models were adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and estimated glomerular filtration rate (eGFR); 2) clinical & time-varying HF medication model: additional adjustment for equivalent doses of carvedilol, enalapril, furosemide, and spironolactone (repeatedly assessed during follow-up) in a time-dependent Cox analysis; 3) established cardiac biomarker model: LME and Cox models were adjusted for the established biomarkers NT-proBNP and high-sensitive troponin T (hsTnT). Results are given as hazard ratios (HR) and 95% confidence intervals (CI) per 1SD difference of the absolute biomarker-candidate level and per 0.1 SD/year difference of the slope at any point in time during follow-up.

We examined a total of 15 serially measured blood biomarkers in relation to the PE (14 marker-candidates of remodeling, plus NT-proBNP). To correct for multiple testing, we performed matrix spectral decomposition.[26,27] Consequently, the corrected significance level was set at $p < 0.005$. We used the conventional $p < 0.05$ threshold to conclude significance for the relation between baseline characteristics and the occurrence of the PE (Table 1), as well as for the relation between first and last biomarker-candidate sample (Table 2). All tests were two-tailed. All analyses were performed with SPSS Statistics 24 (IBM Inc., Chicago, IL) and R Statistical Software using packages nlme[28] and JMbayes.[24] The matrix spectral decomposition application was available online.[29]

RESULTS

Baseline characteristics

Table 1 shows baseline characteristics in relation to the occurrence of the PE. Patients who experienced the PE during follow-up were older, had a lower systolic blood pressure, higher NYHA class and higher levels of NT-proBNP and hsTnT. Furthermore, they more frequently had diabetes and atrial fibrillation, and were more often on diuretics. The majority of the examined biomarker-candidates (ST2, Gal-3, Gal-4, GDF-15, MMP2, TIMP4, PLC, AP-N, CTSZ, CSTB and NTproBNP) showed significantly higher levels at baseline in patients who later experienced the endpoint than in patients who remained event-free (Table 1).

TABLE 1 **Patients characteristics in relation to the primary endpoint.**

Variable	Total	Composite endpoint reached		p-value*
		Yes	No	
n (%)	263 (100)	70 (27)	193 (73)	
Demographics				
Age, years	68 (59-76)	72 (60-80)	67 (58-75)	0.021
Men	189 (72)	53 (76)	136 (71)	0.40
Clinical characteristics				
Body Mass Index, kg/m^2	26 (24-30)	27 (24-30)	26 (24-30)	0.80
Heart rate, beats/min	67 ± 12	69 ± 13	67 ± 11	0.22
Systolic blood pressure, mm Hg	122 ± 20	117 ± 17	124 ± 21	0.020
Diastolic blood pressure, mm Hg	72 ± 11	70 ± 10	73 ± 11	0.055
Features of heart failure				
NYHA class III or IV	69 (26)	31 (44)	38 (20)	< 0.001
HFrEF	250 (95)	66 (94)	184 (95)	0.75
HFpEF	13 (5)	4 (6)	9 (5)	
LVEF	32 ± 10	30 ± 11	33 ± 10	0.18
Established biomarkers				
NT pro-BNP, pmol/L	137 (52-273)	282 (176-517)	95 (32-208)	< 0.001
HsTnT, ng/L	18 (10-33)	32 (21-50)	14 (8-27)	< 0.001
eGFR, ml/min per 1.73m^2	58 (43-76)	53 (40-73)	59 (44-77)	0.20
Etiology of heart failure, n (%)				
Ischemic	117 (44)	36 (51)	81 (42)	0.17
Hypertension	34 (13)	10 (14)	24 (12)	0.69
Valvular disease	12 (5)	5 (7)	7 (4)	0.31
Cardiomyopathy ‡	68 (26)	15 (21)	53 (28)	0.32
Unknown or Others	32 (12)	4 (6)	28 (15)	
Medical history, n (%)				
Prior MI	96 (37)	32 (46)	64 (33)	0.06
Prior PCI	82 (31)	27 (39)	55 (29)	0.12
Prior CABG	43 (16)	13 (19)	30 (16)	0.56
History of ICD implantation	156 (59)	44 (63)	112 (58)	0.48
Prior CVA/TIA	42 (16)	15 (21)	27 (14)	0.15
Atrial fibrillation	106 (40)	36 (51)	70 (36)	0.027
Diabetes Mellitus	81 (31)	32 (46)	49 (25)	0.002
Hypercholesterolemia	96 (37)	30 (43)	66 (34)	0.20

continued

Variable	Total	Composite endpoint reached Yes	No	p-value*
Hypertension	120 (46)	38 (54)	82 (43)	0.090
COPD	31 (12)	12 (17)	19 (10)	0.11
Medication use, n (%)				
Beta-blocker	236 (90)	61 (87)	175 (91)	0.40
ACE-I or ARB	245 (93)	63 (90)	182 (94)	0.22
Diuretics	237 (90)	68 (97)	169 (88)	0.021
Loop diuretics	236 (90)	68 (97)	168 (87)	0.017
Thiazides	7 (3)	3 (4)	4 (2)	0.39
Aldosterone antagonist	179 (68)	53 (76)	126 (65)	0.11
KDOQI classification, n (%)				
eGFR ≥ 90	28 (11)	7 (10)	21 (11)	0.18
eGFR 60-89	95 (36)	20 (28)	75 (39)	
eGFR 30-59	119 (45)	37 (53)	82 (42)	
eGFR < 30	21 (8)	6 (9)	15 (8)	
Biomarker level at baseline in arbitrary unit (linear NPX values)				
ST2	10.36 (7.25-13.65)	12.32 (8.41-17.20)	9.45 (7.05-12.23)	<0.001
Gal-3	38.47 (31.76-46.94)	42.60 (33.68-53.12)	38.20 (31.10-44.71)	0.007
Gal-4	8.90 (6.71-12.61)	12.32 (8.41-17.20)	9.45 (7.05-12.23)	0.001
GDF-15	45.23 (31.52-75.42)	66.01 (41.80-119.28)	41.38 (29.24-61.73)	<0.001
MMP-2	17.63 (14.03-22.67)	19.84 (15.28-27.47)	16.33 (13.09-21.56)	<0.001
MMP-3	76.13 (53.56-105.23)	77.24 (56.71-111.93)	76.10 (53.15-104.45)	0.31
MMP-9	9.10 (6.50-13.67)	9.54 (6.23-15.80)	8.69 (6.54-13.46)	0.45
TIMP4	17.14 (13.09-23.41)	20.89 (14.84-26.17)	16.24 (12.16-22.03)	<0.001
PLC	80.74 (60.76-110.60)	107.61 (73.44-145.58)	73.26 (57.79-98.69)	<0.001
AP-N	22.47 (18.73-28.59)	25.59 (18.68-32.44)	21.73 (18.69-27.28)	0.029
CASP3	262.88 (140.42-490.67)	295.91 (137.09-571.90)	257.34 (142.03-472.55)	0.32
CTSD	32.00 (25.47-41.42)	33.05 (27.18-46.44)	31.89 (24.98-41.05)	0.19
CTSZ	33.02 (26.16-44.45)	37.04 (26.65-49.51)	31.97 (25.9-42.65)	0.039
CSTB	51.12 (36.91-78.66)	76.85 (50.29-103.80)	46.77 (33.68-64.53)	<0.001
NT-proBNP	8.90 (3.82-16.93)	18.48 (11.19-33.71)	6.32 (2.82-12.39)	<0.001

MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; ACE-I, angiotensin-converting enzyme inhibitors; AP-N, aminopeptidase-N; ARB, angiotensin II receptor blockers; CASP3, Caspase-3(CASP3); CSTB, Cystatin-B; CTSD, Cathepsin D; CTSZ, Cathepsin Z; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; Gal-3, galectin-3; Gal-4, galectin-4; GDF-15, growth differentiation factor 15; ICD, Implantable Cardioverter Defibrillator; KDOQI, National Kidney Foundation–Kidney Disease Outcome Quality Initiative; NPX, normalized protein expression;

CVA, cerebrovascular accident; MMP-2, 3 and 9, matrix metalloproteinase 2, 3 and 9; NT-proBNP, N-terminal pro–B-type natriuretic peptide; NYHA class, New York Heart Association class; PLC, Perlecan; ST2, Suppression of tumorigenicity-2; TIA, transitory ischemic attack; TIMP-4, Tissue Inhibitor Metalloproteinase 4. Variables with a normal distribution are presented as mean ± SD, whereas non-normally distributed continuous variables are expressed as median (25^{th} – 75^{th} percentile). Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts, because of missing values. * p value <0.05. ‡ Cardiomyopathy comprised hypertrophic, dilated, restrictive, arrhytmogenic right ventricular, non-compaction cardiomyopathy or unclassified cardiomyopathy.

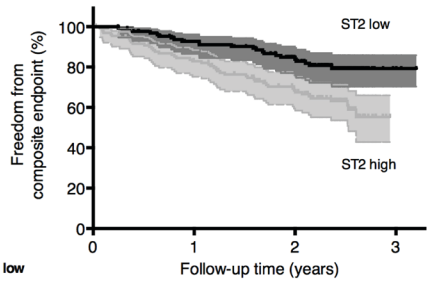
Follow-up and study endpoints

During a median (IQR) follow-up of 2.2 (1.4–2.5) years, we collected 9 (5–10) blood samples per patient. amounting to a total of 1984 samples. After selecting all baseline samples, the two samples closest in time to the composite endpoint, and the last sample available for event-free patients, 567 samples were available for the current investigation.

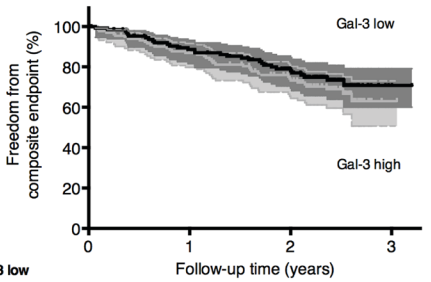
A total of 70 (27%) patients reached the PE: 56 patients were re-hospitalized for acute or worsened HF, 3 patients underwent heart transplantation, 2 patients underwent LVAD placement, and 9 patients died of cardiovascular causes. Overall, freedom from the composite endpoint was 0.76±0.03 at 2 years of follow-up (Figure S1). In particular baseline ST-2 and GDF-15 levels above the median showed worse freedom from composite endpoint (Figure 2).

Temporal patterns of biomarkers in relation to the occurrence of study endpoints

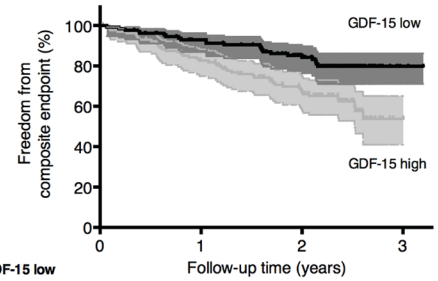
Figure 3 shows the average temporal patterns of cardiac remodeling biomarker-candidates in patients with and without the PE. Twenty-four months before occurrence of the endpoint, ST2 levels were already higher in patients who ultimately reached the PE compared to patients who remained event-free. Furthermore, ST2 significantly increased as the endpoint approached. All biomarker-candidates, except for CASP3 and CTSZ, showed a similar pattern although sometimes less outspoken.



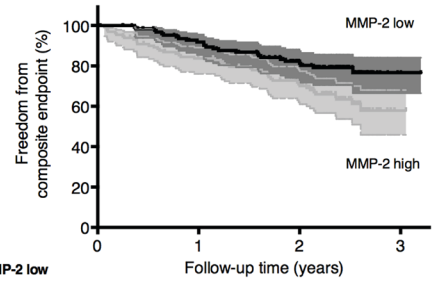
ST2 low				
Number of remaining patients:	130	111	85	54
Estimate:		0.93	0.84	0.79
St. Error:		± 0.02	± 0.03	± 0.04
ST2 high				
Number of remaining patients:	131	105	74	15
Estimate:		0.83	0.69	0.55
St. Error:		± 0.03	± 0.04	± 0.06



Gal-3 low				
Number of remaining patients:	130	105	79	27
Estimate:		0.89	0.78	0.71
St. Error:		± 0.03	± 0.04	± 0.05
Gal-3 high				
Number of remaining patients:	131	109	80	17
Estimate:		0.87	0.74	0.63
St. Error:		± 0.03	± 0.04	± 0.06



GDF-15 low				
Number of remaining patients:	130	114	86	72
Estimate:		0.93	0.84	0.80
St. Error:		± 0.02	± 0.03	± 0.04
GDF-15 high				
Number of remaining patients:	131	102	73	14
Estimate:		0.83	0.68	0.54
St. Error:		± 0.03	± 0.04	± 0.06



MMP-2 low				
Number of remaining patients:	130	109	80	30
Estimate:		0.92	0.81	0.77
St. Error:		± 0.03	± 0.04	± 0.05
MMP-2 high				
Number of remaining patients:	131	105	79	17
Estimate:		0.84	0.71	0.58
St. Error:		± 0.03	± 0.04	± 0.06

FIGURE 2 Freedom from the composite endpoint for ST2, Gal-3, GDF-15 and MMP-2 above and below the median value.

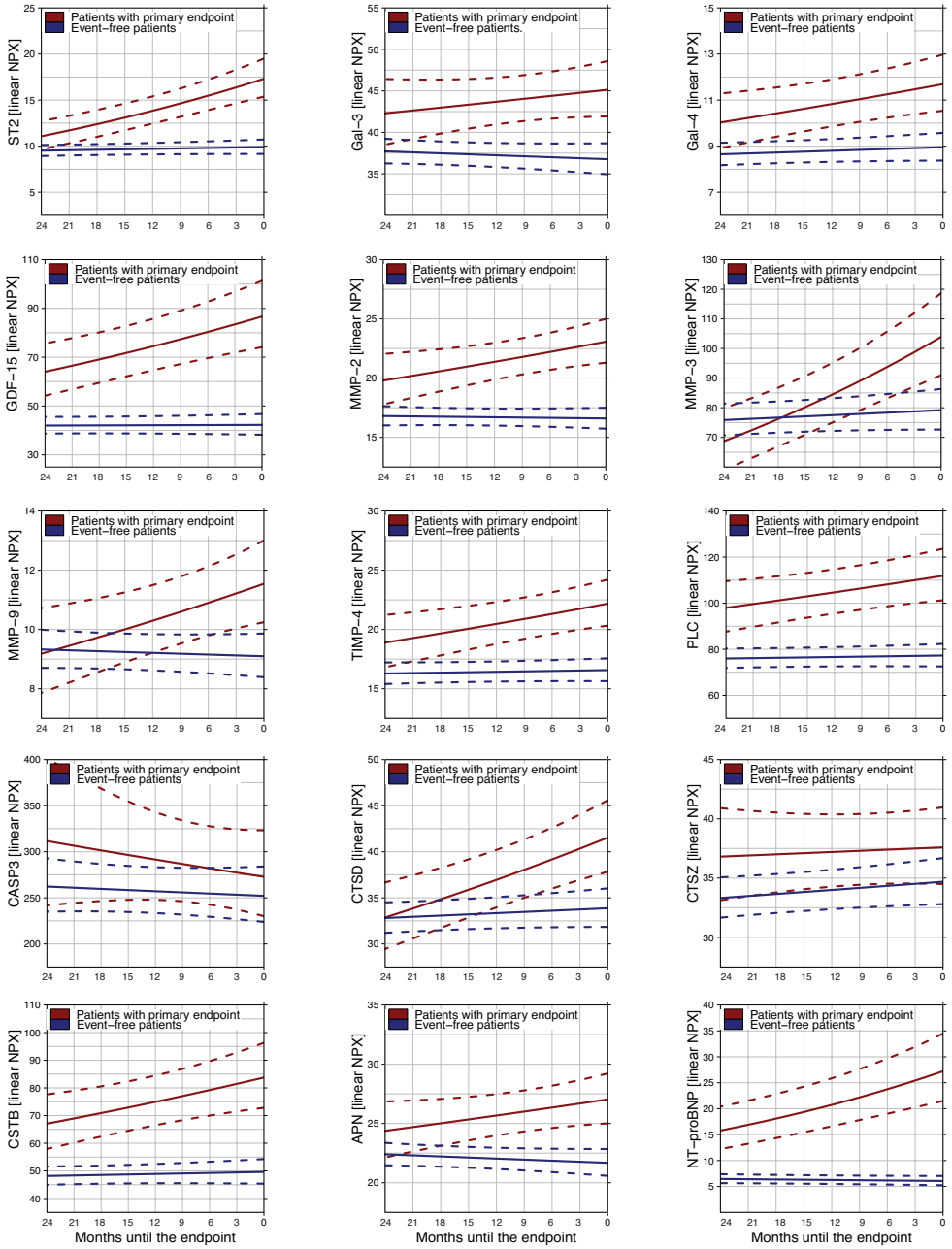


FIGURE 3 Average temporal patterns of cardiac remodeling biomarker-candidates during follow-up. X-axis: time remaining to the primary endpoint (for patients who experienced incident adverse events) or time remaining to last sample moment (for patients who remained event-free). Of note is that 'time zero' Is defined as the occurrence of the endpoint and is depicted on the right side of the x-axis, so that the average marker trajectory can be visualized as the endpoint approaches. Y-axis: biomarker

levels in arbitrary, relative units (normalized protein expression, NPX on linear scale). Solid red line: Average temporal pattern of biomarker-candidate level in patients who reached the primary endpoint during follow-up. Solid blue line: Average temporal pattern of biomarker-candidate level in patients who remained endpoint free. Dashed lines: 95% confidence interval. AP-N, aminopeptidase-N; CASP3, Caspase-3; CSTB, Cystatin-B; CTSD, Cathepsin D; CTSZ, Cathepsin Z; Gal-3, galectin-3; Gal-4, galectin-4; GDF-15, growth differentiation factor 15; MMP-2, 3 and 9, matrix metalloproteinase 2, 3 and 9; NPX, Normalized Protein Expression; NT-proBNP, N-terminal pro–B-type natriuretic peptide; PLC, Perlecan; ST2, Suppression of tumorigenicity-2; TIMP-4, Tissue Inhibitor Metalloproteinase 4.

Table 3 shows the associations of cardiac remodeling biomarker-candidates with the PE. After adjustment for clinical characteristics, as well as after additional adjustment for HF medication doses during follow-up, ST2 was the numerically strongest predictor of the PE (HR 7.55 per 1 SD difference, 95%CI 5.53-10.30), followed by GDF-15 (HR 4.06, 95%CI 2.98-5.54) and MMP-2 (HR 3.59, 95%CI 2.55-5.05). Moreover, Gal-3, Gal-4, MMP-3 and 9, TIMP-4, PLC, AP-N, CTSD, CSTB, and NT-proBNP independently predicted the endpoint (all p-values <0.005). Furthermore, levels of these biomarker-candidates, except for MMP-3 and AP-N, remained significant predictors after adjusting for cardiac markers NT-proBNP and hsTnT.

Independently of their levels, the slopes (rates of change over time) of ST2, Gal-3, Gal-4, GDF-15, MMP-2, 3 and 9, TIMP-4, PLC, CTSD, and NT-proBNP remained significant predictors after adjusting for clinical characteristics and HF medication (clinical and time-varying medication model), as well as after adjustment for established cardiac biomarkers (established cardiac biomarker model, latter except for Gal-4 and MMP-3) (p-values <0.005, for HR see Table 4).

DISCUSSION

In this prospective repeated-measures study in 263 patients with stable CHF, we demonstrated that levels of biomarker-candidates of cardiac remodeling (such as ST2, Gal-3, Gal-4, GDF-15, MMP-2 and 9, TIMP-4, PLC, CTSD and CSTB) increase markedly and significantly as an adverse clinical event approaches. Importantly, their repeatedly measured levels strongly predict incident adverse clinical events with ST2 being the strongest predictor. Independently of their levels, the rate of biomarker change over time of these biomarker-candidates also predicts incident events. These associations persist after multivariable adjustment for clinical characteristics, pharmacological treatment during follow-up, and established cardiac biomarkers NT-proBNP and hsTnT.

TABLE 3 **Associations between blood biomarkers of cardiac remodeling and the primary endpoint – levels.**

	Crude model		Clinical model		Clinical and time-varying medication model		Cardiac biomarker model	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Level (per SD difference)								
ST2	5.63 (3.67-10.15)	<0.001*	5.93 (3.67-11.67)	<0.001*	7.55 (5.53-10.30)	<0.001*	4.02 (2.56-7.07)	<0.001*
Gal-3	1.91 (1.43-2.58)	<0.001*	2.11 (1.50-2.99)	<0.001*	3.23 (2.32-4.48)	<0.001*	1.57 (1.22-2.03)	<0.001*
Gal-4	1.92 (1.46-2.51)	<0.001*	1.68 (1.23-2.29)	<0.001*	2.11 (1.57-2.84)	<0.001*	1.54 (1.15-2.05)	<0.002*
GDF-15	3.09 (2.39-4.15)	<0.001*	3.11 (2.25-4.40)	<0.001*	4.06 (2.98-5.54)	<0.001*	2.50 (1.83-3.48)	<0.001*
MMP-2	3.17 (2.27-4.61)	<0.001*	3.21 (2.06-5.31)	<0.001*	3.59 (2.55-5.05)	<0.001*	2.45 (1.66–3.75)	<0.001*
MMP-3	1.60 (1.25-2.04)	0.001*	1.46 (1.08-1.95)	0.019	1.77 (1.35-2.32)	<0.001*	1.22 (0.93-1.61)	0.153
MMP-9	1.87 (1.32-2.69)	0.001*	1.75 (1.23-2.54)	<0.001*	2.53 (1.82-3.52)	<0.001*	1.75 (1.24-2.49)	<0.001*
TIMP-4	2.55 (1.83-3.61)	<0.001*	2.45 (1.65-3.81)	<0.001*	2.95 (2.13-4.09)	<0.001*	1.69 (1.21-2.40)	<0.001*
PLC	2.66 (1.98-3.60)	<0.001*	2.58 (1.76-3.88)	<0.001*	2.66 (1.96-3.62)	<0.001*	1.89 (1.32-2.73)	<0.001*
AP-N	2.04 (1.51-2.77)	<0.001*	1.75 (1.30-2.38)	<0.001*	1.83 (1.35-2.49)	<0.001*	1.53 (1.15-2.03)	0.005
CASP3	1.15 (0.83-1.58)	0.41	x		x		x	
CTSD	1.76 (1.37-2.28)	<0.001*	1.73 (1.26-2.35)	0.001*	1.80 (1.38-2.35)	<0.001*	1.67 (1.29-2.19)	<0.001*
CTSZ	1.37 (1.06-1.77)	0.023	x		x		x	
CSTB	2.10 (1.69-2.63)	<0.001*	2.39 (1.74-3.24)	<0.001*	2.93 (2.20-3.90)	<0.001*	1.70 (1.33-2.19)	<0.001*
NT-proBNP	4.50 (3.30-6.25)	<0.001*	4.35 (3.13-6.31)	<0.001*	4.80 (3.43-6.70)	<0.001*	4.27 (3.04-6.17)	<0.001*

HR (95% CI) are given per SD increase at any point in time during follow-up. **Crude model**: Cox model unadjusted, linear mixed effect (LME) model adjusted for sampling time; **Clinical model**: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, eGFR, and sampling time (LME); **Clinical and time-varying medication model**: Time-dependent Cox additionally adjusted for total daily doses carvedilol, enalapril, furosemide, and spironolactone during follow-up; **Cardiac biomarker model**: Cox and LME models adjusted for baseline NT-proBNP and hsTnT, and sampling time (LME). x not performed because repeatedly measured level was not significant; * p-value below the corrected significance level for multiple testing (p-value <0.005).

ST2 is an interleukin-1 receptor family member and an increase of the soluble circulating form of ST2 promotes myocardial apoptosis, fibrosis, and hypertrophy.[30] Higher ST2 plasma concentrations have shown to be among the strongest predictors of adverse outcome in CHF such as worsening HF and risk for either hospitalization or death from HF.[30] Accordingly, the updated guidelines for the management of HF suggest the use of ST2 for risk stratification in CHF patients.[31] In line with this, our study shows that ST2 is the biomarker-candidate whose association with adverse events is numerically the strongest out of the studied 14 biomarker-candidates of cardiac remodeling. Previously, several studies have examined the prognostic value of repeatedly measured ST2[10-12], but certain limitations restricted their generalizability. One study had a relatively short follow-up period of 10 months after recent HF decompensation[11], other studies re-measured ST2 infrequently (only in the beginning of the follow-up without regular measurements during the remaining follow-up), with clinical events occurring outside the sampling window.[10,12] Using such approaches, a relatively long time interval is left between the last ST2 measurement and the adverse event that occurs eventually. This may distort potential associations considering that CHF is a dynamic disease, and the levels of the biomarkers that reflect the underlying disease process may be expected to change as the adverse event approaches.[9] Ideally, the time interval between the last biomarker measurement and the adverse event should be kept as brief as possible in order to investigate accurately whether ST2 levels increase shortly before an adverse event and whether this increase truly contributes to the patient's risk. Another limitation is that the rate of change in ST2 might not be properly captured in former studies, as changes are often described as the difference between any two measurements without incorporating the time interval during which these changes occurred. In this way, the temporal biomarker pattern that occurs when an event is approaching is not taken into account, although this may in fact be of most value in individual risk prediction.

Our study extends current knowledge while addressing previous limitations, as we have performed repeated blood sampling based on a pre-specified study protocol at fixed three-month intervals over the full course of follow-up, with up to 11 samples per patient. This enabled us to select the two samples closest to an adverse event for our analyses. We show not only that ST2 levels differ at baseline between patients with and without incident events, but, importantly, we also demonstrate an increase in ST2 level as an adverse event approaches. Another unique finding is that the rate of the ST2 change over time independently predicts adverse clinical outcome. In other words, prognosis differs between patients who have high and stable ST2 levels and patients with high but rapidly increasing ST2 levels, which additionally underlines the incremental value of serial ST2 measurements.

TABLE 4 **Associations between blood biomarkers of cardiac remodeling and the primary endpoint – slopes.**

	Crude model		Clinical model		Clinical and time-varying medication model		Cardiac biomarker model	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Slope (per 0.1 SD/year difference)								
ST2	1.14 (1.08-1.21)	<0.001*	1.21 (1.11-1.34)	<0.001*	1.13 (1.11-1.16)	<0.001*	1.12 (1.06-1.18)	<0.001*
Gal-3	1.22 (1.11-1.36)	<0.001*	1.45 (1.19-1.88)	<0.001*	1.13 (1.07-1.19)	<0.001*	1.13 (1.05-1.22)	<0.001*
Gal-4	1.18 (1.05-1.36)	0.004*	1.27 (1.09-1.70)	<0.001*	1.10 (1.03-1.18)	<0.001*	1.12 (1.03-1.23)	0.015
GDF-15	1.22 (1.13-1.33)	<0.001*	1.29 (1.17-1.46)	<0.001*	1.12 (1.09-1.15)	<0.001*	1.18 (1.00-1.27)	<0.001*
MMP-2	1.19 (1.08-1.32)	<0.001*	1.29 (1.10-1.68)	<0.001*	1.18 (1.10-1.26)	<0.001*	1.14 (1.06-1.24)	<0.002*
MMP-3	1.16 (1.06-1.34)	0.002*	1.20 (1.04-1.57)	0.008	1.09 (1.04-1.15)	<0.001*	1.09 (1.02-1.19)	0.026
MMP-9	1.29 (1.19-1.41)	<0.001*	1.46 (1.27-1.76)	<0.001*	1.17 (1.12-1.23)	<0.001*	1.20 (1.12-1.29)	<0.001*
TIMP-4	1.31 (1.21-1.43)	<0.001*	1.03 (0.73-1.63)	0.25	1.16 (1.10-1.22)	<0.001*	1.23 (1.15-1.34)	<0.001*
PLC	1.26 (1.12-1.47)	<0.001*	1.37 (1.16-1.75)	<0.001*	1.13 (1.05-1.22)	0.002*	1.20 (1.08-1.33)	<0.001*
AP-N	1.16 (1.03-1.33)	0.010	x		x		x	
CASP3	0.84 (0.72-0.97)	0.018	x		x		x	
CTSD	1.27 (1.16-1.42)	<0.001*	1.47 (1.25-1.85)	<0.001*	1.15 (1.09-1.22)	<0.001*	1.16 (1.08-1.26)	<0.001*
CTSZ	1.21 (0.81-1.42)	0.16	x		x		x	
CSTB	1.00 (0.88-1.12)	0.97	x		x		x	
NT-proBNP	1.21 (1.10-1.36)	<0.001*	1.33 (1.15-1.63)	<0.001*	1.21 (1.16-1.27)	<0.001*	1.18 (1.08-1.30)	<0.001*

Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 0.1 SD of the annual slope at any point in time during follow-up. Annual slopes were additionally adjusted for the levels of repeatedly measured marker during follow-up. For adjusted models and abbreviations please see description under Table 3.

x not performed because repeatedly measured level was not significant; * p-value below the corrected significance level for multiple testing (p-value <0.005).

Gal-3 is a soluble β-galactoside-binding lectin and a member of the galectin family[32] and this biomarker is deemed a relevant mediator in the cardiac remodeling process.[33] A recent meta-analysis showed that increased Gal-3 levels carry higher risk of mortality independently of well-established risk factors.[34] Nevertheless, whether this association between Gal-3 and adverse outcome is independent of natriuretic peptides remained unclear.[35-37] In addition, studies on repeatedly measured Gal-3 are scarce. Our results show that repeatedly measured Gal-3 levels increase over time as an adverse event approaches, and that these levels significantly predict adverse clinical events even after multivariable adjustment that included NT-proBNP. These findings are also supported by Van der Velde et al., who showed that Gal-3 is of significant prognostic value in identifying high-risk CHF patients after combining data from the CORONA trial (baseline measurement plus additional measurement after 3 months) and the COACH trial (baseline measurement plus additional measurement after 6 months).[9] Less is known about Gal-4, another member of the galectin family. Although its physiological and pathophysiological features still need clarification, our results suggest that Gal-4 might be a promising biomarker in CHF patients since its level, as well as its change over time, showed a strong association with the PE.

In pathological conditions, GDF-15, a remote member of the transforming growth factor-β (TGF-β) super family, may influence cardiac remodeling via two different mechanisms, i.e., protection from apoptosis and induction of hypertrophy.[38] Several studies have shown promising results on the prognostic value of GDF-15. Chan et al.[39] found prognostic utility of GDF-15 measured at 6 weeks and 5 months beyond NT-proBNP in both HF patients with reduced ejection fraction (HFrEF) and those with preserved ejection fraction (HFpEF). In the HF-ACTION Study (HFrEF patients)[7], GDF-15 provided independent prognostic information incremental to hsTnT and NT-proBNP. Our results support and extend these findings by demonstrating that repeatedly measured levels of GDF-15, together with ST2, MMP-2 and NT-proBNP, show the numerically strongest independent associations with the PE (also after multivariable adjustment).

Biomarkers of cardiac extracellular matrix turnover include MMPs, their inhibitors (TIMPs), and the less studied PLC and AP-N. Several MMPs and TIMPs are associated with fibrosis, diastolic dysfunction and left ventricular hypertrophy[40,41], and some of these, such as MMP-9 and TIMP-1, correlated with the severity of CHF[42]. Moreover MMPs are implicated in several cardiovascular diseases; for example MMP-2 and -9 are potential biomarkers of acute myocardial infarction43 and coronary artery disease.[44] Furthermore, MMP-2 may be most suitable for serial biomarker measurements, as suggested by Täger et al. who performed

multiple measurements over a time span of 2 weeks of MMP-2, MMP-9, TIMP-1, and TIMP-4 in 50 patients with CHF.[45] In our study MMP-2, MMP-9, TIMP-4 and PLC were clear predictors of the PE. Conversely, level and slope of MMP-3 was not a significant predictor of adverse events after adjustment. AP-N is a type II metalloprotease[46], which is relatively unknown in the field of cardiac diseases. Although AP-N level was a strong predictor of the PE in our study, the rate of change over time (i.e., slope) was not. These results suggest that repeated measurement of AP-N may be unnecessary for prognostication, and single measurement may suffice.

Little or no data is available on biomarkers of apoptosis, like CASP3, CTSD, CTSZ and CSTB, and their role in cardiac remodeling and CHF prognosis. However, apoptosis has been investigated as a pathophysiologic mechanism in CHF. Since this study demonstrates interesting results regarding the prognostic value of the level of CSTB and both level and slope of CTSD, further investigations of the role of these novel biomarker-candidates in CHF should be encouraged.

Of interest, patients in the current study were in a better health condition than previously reported CHF populations since 74% was in NYHA class I-II. Still, we were able to show that biomarker-candidates of cardiac remodeling are strongly associated with clinical outcome. These findings raise the hypothesis that this NYHA class I-II patient group in particular may benefit from serial measurements of the studied biomarkers for prognostication, and ultimately to guide therapeutic interventions in order to prevent progression to advanced stage disease.

Study limitations

Our study carries several limitations. Firstly, as described before[16], our cohort comprised mainly HFrEF patients. This can most likely be attributed to the fact that in the Netherlands, most HFpEF patients are followed in secondary referral centers or by the general practitioner, while the current study was performed in two tertiary referral centers. Secondly, although we had trimonthly blood samples available for all patients, because of efficiency reasons 2 sampling moments were selected for event-free patients, and 3 sampling moments for patients with a PE. In previous investigations in this cohort[15], we have used all available sampling moments to determine NT-proBNP, hsTnT, C-reactive protein (CRP) as well as glomerular and tubular renal biomarkers.[16] Those investigations demonstrated that most of these biomarker-candidates show an increase shortly prior to the incident adverse event. Thus, we believe that by selecting baseline samples, as well as the last 2 samples prior to the incident endpoint, we retain the most informative measurements while enhancing efficiency. Finally, the assay we used for measuring the biomarker-can-

didates was designed as a biomarker discovery tool rather than being an approved clinical test. Future research should investigate standardization of the assays in order to successfully translate these emerging biomarkers into daily clinical practice.

CONCLUSION

This study shows that temporal patterns of patient-specific levels of numerous biomarker-candidates of cardiac remodeling strongly predict clinical outcome in CHF; specifically, these remodeling biomarker-candidates increase prior to an adverse event in CHF patients. These patient-specific temporal patterns indicate a promising role of these biomarker-candidates for individual prognostication and treatment monitoring.

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SUPPLEMENTARY INFORMATION

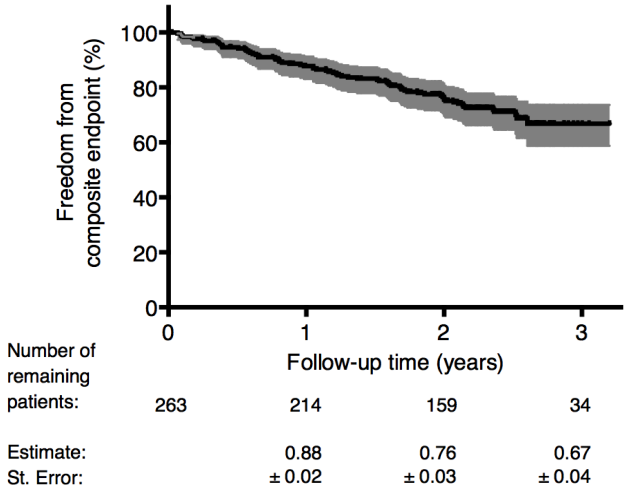
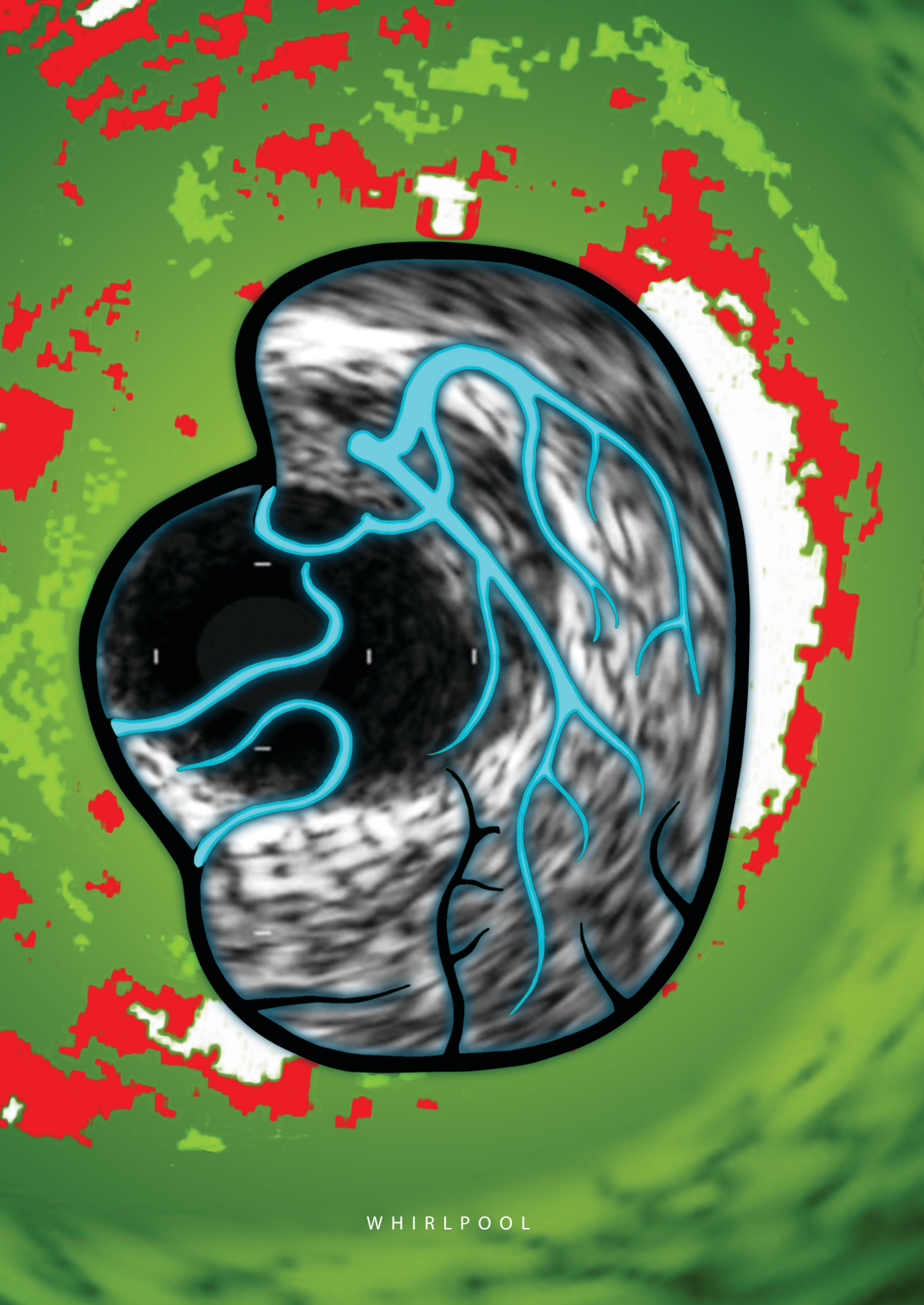


FIGURE S1 Freedom from the composite endpoint.


WHIRLPOOL

Part III

IMPLICATIONS OF RENAL FUNCTION FOR ISCHEMIC HEART DISEASE

CHAPTER 9

Evolution of Renal Function and Predictive Value of Serial Renal Assessments among Patients with Acute Coronary Syndrome - The BIOMArCS study

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Submitted

ABSTRACT

Background

Renal dysfunction predicts mortality in acute coronary syndrome (ACS), but its evolution following and preceding ACS has never been described in detail. We aimed to describe this evolution, quantified by creatinine, estimated glomerular filtration rate ($eGFR_{Cr}$), and cystatin C (CysC), from its initial change during ACS until stabilization; and to investigate the predictive value of serial assessments of these renal markers in patients with ACS.

Methods

From 844 ACS patients included in the BIOMArCS study, we analysed a case-cohort consisting of 187 (random sample of 150 patients, plus all those who reached the endpoint) to determine the risk of the composite endpoint (cardiovascular death or hospitalization for non-fatal ACS) in relation to marker levels and their rates of change during 1-year follow-up. In each patient, the marker trajectories were estimated using repeated measurements (mean 8 per patient). Survival analyses were adjusted for GRACE risk score, and based on all available data >30 days after the index ACS to ensure stabilization of renal markers.

Results

Mean age was 63 years, 79% were men, 43% had STEMI, and 67% were in CKD stages 2-3. During hospitalization (median[IQR] duration: 5 [3-7] days), CysC levels indicated deterioration of renal function earlier than creatinine did (CysC peaked on day 3, versus day 6 for creatinine), and stabilized after two weeks. Higher CysC levels predicted the endpoint independently of the GRACE score (per 1SD increase: adjusted HR [95%CI]: 1.68 [1.03–2.74]). However, the rates of CysC change were not significant predictors.

Conclusions

CysC levels are the earliest indicators of deterioration of renal function, which usually does not stabilize during hospitalization, but on average two weeks after index ACS. In ACS patients with normal to moderately impaired renal function, after stabilization of renal function, CysC levels predict adverse events within the first year.

INTRODUCTION

Renal dysfunction, including mild renal impairment ($eGFR_{Cr}$ 60-89 ml/min/1.73m^2),[1,2] is strongly associated both with short- and long-term mortality in patients with ST elevation myocardial infarction (STEMI) and in those with non-STEMI.[3-5] Patients with chronic kidney disease (CKD) are often treated less aggressively for acute coronary syndrome (ACS) than those without CKD.[3,4,6] However, even if they are on optimal therapy they will still have poorer prognosis.[7] Renal dysfunction is associated both with coronary atherosclerosis, including higher coronary plaque burden, plaques containing greater necrotic core and more dense calcium, as well as with abnormalities of cardiac muscle, including left ventricular hypertrophy, dilated cardiomyopathy, and systolic dysfunction.[8-10] Several studies have shown that specific comorbidities such as hypertension, diabetes, and dyslipidemia, contribute both to cardiovascular and renal damage.[11,12] Neuro-hormonal activation is also affected after ACS,[13-15] and angiotensin II may influence deterioration of both cardiovascular and renal functioning.[13,16,17]

In spite of these overlapping pathophysiological aspects, the detailed temporal evolution of renal function immediately following ACS, and preceding a recurrent ACS, has not yet been described. Existing studies have mostly assessed renal function only at a single time point to investigate its prognostic value, and have used for example time of admission, a moment during in-hospital stay or time of discharge as 'study baseline'. However, it is unclear whether a patient's renal function examined at these time points during hospitalization reflects "true" renal functioning or whether it is temporarily disturbed by the index ACS. Moreover, it remains unknown at which moment after ACS renal function stabilizes. Knowing these temporal patterns may help us in expanding our understanding of renal dysfunction in patients with ACS, and thereby aid in identifying high-risk subgroups.

The aim of our study was two-fold: (1) to describe the evolution of renal function from its initial change during ACS until stabilization, according to the kinetics of several renal function parameters (plasma creatinine, estimated glomerular filtration rate [eGFR], and cystatin C [CysC]), (2) to investigate the predictive value of serial renal assessments within the first year after index ACS. For the latter purpose, we also examined whether rates of change of these renal markers are relevant for clinical risk prediction.

METHODS

BIOMArCS is a multi-centre prospective study conducted in 18 Dutch hospitals. Details on the BIOMArCS design are reported elsewhere.[18] Briefly, we included patients who were hospitalized for ACS including STEMI, non-STEMI, and unstable angina pectoris (UAP), with ≥1 cardiovascular risk factor (Table S1). $eGFR_{Cr}$ <30 ml/min/1.73m^2 was an exclusion criterion because of the potential influence of renal clearance on certain biomarkers investigated in the BIOMArCS cohort. Of 844 enrolled patients, 45 reached the study endpoint during a median (IQR) follow-up of 11.5 (2.7–12.1) months.

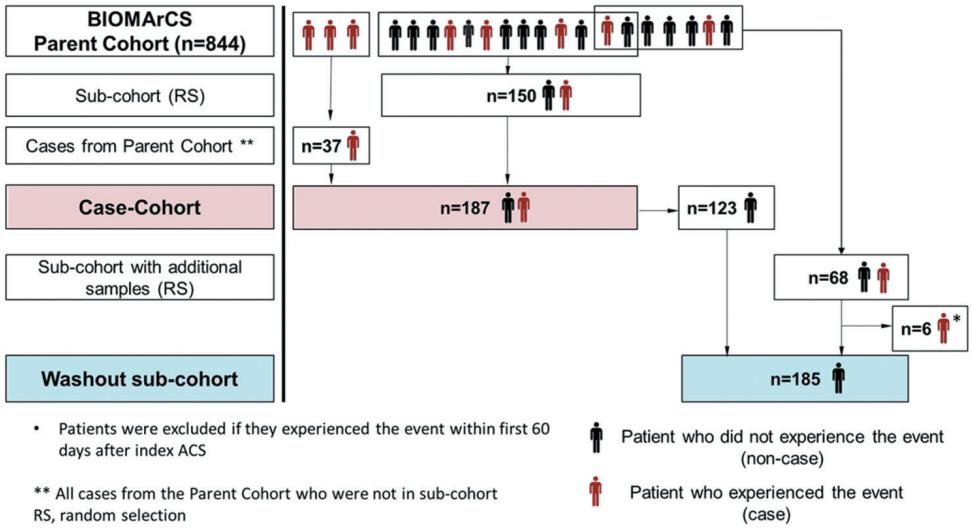
All patients were treated according to prevailing guidelines and at the discretion of the treating physician. The study protocol has been approved by the Institutional Review Board of all participating hospitals and written informed consent was obtained from all patients.

Selection of patients to analyse the relation between renal markers and repeat ACS

For the analysis of the relation between (renal) biomarkers and repeat ACS during 1-year follow-up, we applied a case-cohort design, which allowed a comparison of all study endpoint cases to a limited random sample of non-cases (instead of all non-cases), thereby increasing the study's efficiency.[19] For this purpose, after study completion (i.e., inclusion, follow-up, and study endpoint adjudication) a sub-cohort of 150 patients was randomly sampled from the parent cohort (n=844), using a computer generated random sampling procedure. Subsequently, all patients who experienced the endpoint, but who were not a part of the random sub-cohort were added (37 cases), so that the case-cohort comprised 187 patients (Figure 1). Thus, we analysed all cases, but analyzed only those non-cases (non-endpoint patients) who were present in the random sub-cohort.

Selection of patients to analyse the washout of renal markers after ACS admission

To enable a precise description of early washout biomarker patterns, a total of 68 (8%) BIOMArCS patients underwent additional blood sampling at 24, 48, 72 and 96 hours after the index ACS. We excluded the 6 patients who experienced the study endpoint, and we added the endpoint-free patients from the random sub-cohort. Thus, a total of 185 patients were available for the analysis of washout patterns of renal biomarkers (Figure 1).



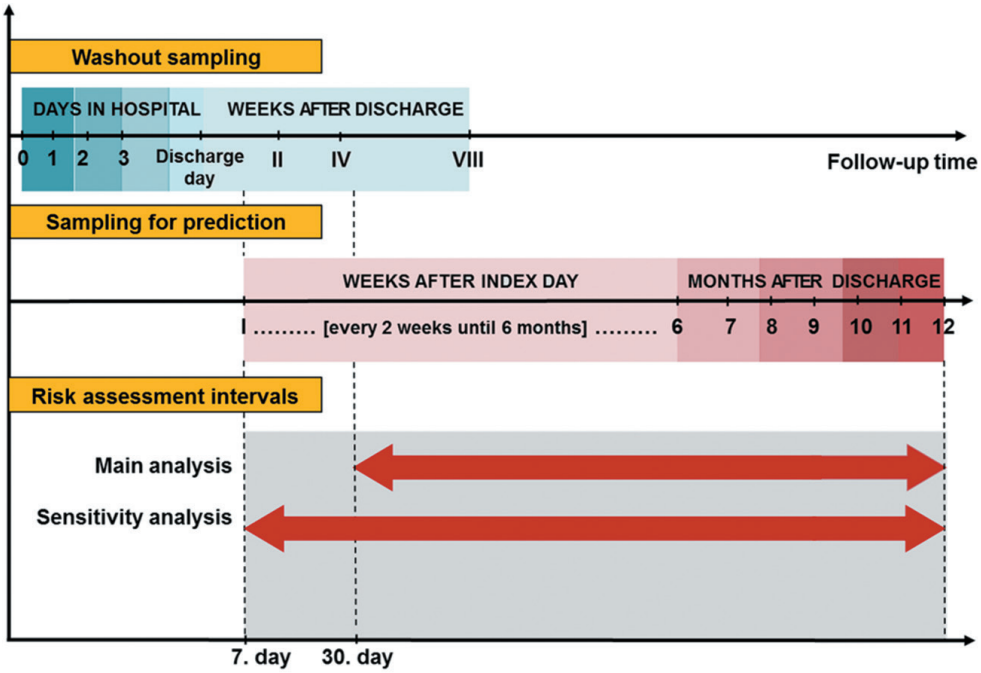


FIGURE 1 **Participants flow chart, study design, and sampling schema.**

Follow-up visits and blood sample collection

Blood samples were collected at admission, hospital discharge, and every two weeks after index ACS during the first six months, followed by monthly collection until one year (Figure 1). A visit window of ±1 week was allowed, and a maximum of two consecutive visits were allowed to be skipped (for personal reasons). If logistic reasons hindered inclusion during hospitalisation, patients could be included on the first outpatient visit within six weeks after discharge; the sampling schedule was then adapted accordingly. A trained research nurse interviewed the patients at each visit and obtained data on anginal status (Canadian Cardiovascular Society classification), heart failure symptomatology (New York Heart Association classification), and factors that might influence biomarker levels, e.g. smoking, occurrence of infections, inflammatory or allergic responses, alterations in medication, interventional or operative procedures and hospital admission. Blood samples were processed on-site and transported batch-wise under controlled conditions (at -80^{0}C) to the department of Clinical Chemistry of the Erasmus MC, Rotterdam where they were stored until analysis was performed.

Glomerular filtration rate (GFR) was determined by the Modification of Diet in Renal Disease (MDRD) Study equation.[20] Patients were categorized using the modified eGFR definition from the National Kidney Foundation – Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines.[21]

Analysis of renal markers

In the 187 case-cohort patients and in the 185 patients that comprise the washout analysis set, renal biomarkers (creatinine and CysC) were measured batch-wise at the laboratory of the department of Clinical Chemistry and Hematology of the University Medical Center Utrecht. Creatinine was measured on clinical routine equipment (AU5800, Beckman Coulter, Brea, CA, USA). Cystatin C was measured by ELISA following manufacturer's instructions (mouse-anti human DuoSet DY1196, R&D Systems, Oxon, UK). Importantly, laboratory personnel were blinded to any patient data and scope of the study, whereas biomarker measurements did not interfere with treatment.

Study endpoints

The study endpoint was a composite of cardiac mortality or a diagnosis of a non-fatal myocardial infarction or unplanned coronary revascularization due to progressive angina pectoris during 1-year follow-up. Any death was considered cardiac unless documented otherwise. Incident non-fatal myocardial infarction was defined as the combination of typical ischemic chest complaints and objective evidence of myocardial ischemia or myocardial necrosis as demonstrated by the ECG and/or elevated cardiac markers. The criteria for non-fatal myocardial infarction during follow-up were the same as those for the index event (Table S1, points 1 and 2 of the inclusion criteria). The Clinical Event Committee, blinded for the renal biomarker results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

Statistical analysis

Case-Cohort – prediction of events

Categorical baseline data are summarized by percentages, and continuous data by medians and 25^{th}–75^{th} percentiles. Differences between cases and non-cases were evaluated by classical statistical tests, as specified in the caption of Table 1.

To obtain valid inferences for the relation between the temporal evolvement of a biomarker and the incidence of the study endpoint, the longitudinal- and event-processes must be jointly modelled. We applied Bayesian semiparametric joint models for this purpose, which combine linear regression and Cox proportional hazard regression. Linear mixed-effects (LME) models were used to describe patient-specific longitudinal biomarker trajectories B(t) as a function of time (t). Non-linear trajectories were modelled by cubic splines. ^{2}Log-transformations of biomarker values were used to assure normal distributions of regression residuals. More specifically, the unit of analysis was the Z-score (i.e., the standardized form) of the ^{2}log-biomarker, which allows a direct comparison of the effects of separate markers. Results are presented as hazard ratios (HR) and corresponding 95% confidence intervals (CI) for a 1SD difference of the biomarker on the log-scale.

The LME models not only provide unbiased estimates B(t) of the biomarker level at timepoint t, but also of its instantaneous rate of change (or: slope) B'(t) at t, that corresponds to the first derivative of B(t). Since we also aimed to study rate of change, we also provided HRs for the instantaneous slope of the marker's trajectory. Results are presented as HRs (95% CIs) for a 0.1SD difference of the marker's rate of change on the log-scale.

Analyses were first performed univariably, and subsequently multivariable adjustment was performed. For this purpose, the GRACE risk score for assessment of post-discharge death and myocardial infraction, as recommended by international guidelines,[22-24] was used. This specific GRACE risk model consists of age, troponin (or CKMB) elevation at admission, history of MI, congestive heart failure and whether CABG was performed at the index hospitalization.[25] The survival model was adjusted for the GRACE risk score, and the LME model was adjusted for GRACE risk score, sex, diabetes, history of coronary artery bypass surgery, history of valvular heart disease, history of stroke, history of peripheral arterial disease.

To describe the average evolution of renal function during the year preceding death or the recurrence of ACS, we analyzed all available data >30 days after the index ACS until the endpoint or last sample moment.

To investigate the predictive value of repeatedly measured markers, we analysed all available data >30 days after the index ACS event, to ensure that all biomarkers were then stabilized. Additionally, a sensitivity analysis was performed on all repeated measurements >7 days after the index ACS. Measurements that were obtained within 7 days after index ACS were excluded to avoid biased estimates due to elevated biomarkers induced by the index ACS.

Analysis of evolution of renal function during the washout phase

LME models were applied to investigate at which time point the renal markers reach their highest point (creatinine, CysC) or lowest point ($eGFR_{Cr}$) and at which time point they return to stable levels. All renal biomarkers were ^{2}log transformed, and non-linear evolutions (for the fixed- and random-effects parts) were modelled by restricted cubic splines. We optimized the position of the spline knots by using Akaike information criteria (AIC) and Bayesian information criteria (BIC). After obtaining optimal evolution curves representing the washout patterns of the renal markers, we calculated the maximum or minimum of these curves to determine the time point of the peak or nadir. To determine the moment of marker stabilization, we also numerically compared the deltas of biomarkers between every two consecutive blood samples (a difference <1% signified a stabilization).

R statistical software (version 2.15.0) was used for advanced statistical analyses, in particular the package JMbayes.[14] All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics, Follow-up, and Study endpoints

Baseline characteristics of all patients in the BIOMArCS study and in the case-cohort set are shown in Table 1. In the case-cohort, on admission mean (±SD) age was 63 (±11) years, 79% were men, 43% had STEMI, 42% had non-STEMI, and 15% had UAP. The median (IQR) eGFR was 81 (70–98) mL/min/1.73m^2, and 33% of patients were in CKD stage 1 (GFR ≥90), 56% in CKD stage 2 (GFR 60–89), and 11% in CKD stage 3 (GFR 30–59).

Average evolutions of renal markers during the washout phase

A total of 687 samples were drawn from the 185 non-endpoint patients that comprise the washout analysis set, with a mean of 4 samples per patient. Average washout evolutions of plasma creatinine, eGFRCr and CysC are shown in Figure 2. The figure shows that CysC levels reached a peak on the 3rd day after index ACS. This was followed by a nadir of eGFRCr on the 4th day, and a peak of creatinine levels on the 6th day. We also found different time intervals from the highest or lowest point to stabilization for these markers: CysC – 11 days (stabilized on day 13) , eGFRCr – 10 days (stabilized on day 13) and creatinine – 8 days (stabilized on day 14). Nevertheless, the stabilization of the markers after index ACS appeared to be temporary. Thereafter, levels continued to steadily change during follow-up (Figure 2 and 3).

Average evolutions of renal markers during the year preceding death or the recurrence of ACS

In the time-period >30 days after index ACS, a total of 1117 blood samples were collected from 158 of the 185 patients that comprise the case-cohort, with a mean of 7 samples per patient – the remaining 27 patients (17 study endpoint cases) only had samples in the 0–30 day time window. Although plasma creatinine levels increased slightly prior to the incident event in patients who ultimately reached the study endpoint, substantial overlap was present between average evolutions of these patients and those who remained endpoint-free (Figure 3). $eGFR_{Cr}$ displayed similar dynamics, but with a smaller overlap. Notably, plasma CysC showed substantially higher levels during follow-up in patients ultimately reaching the study endpoint.

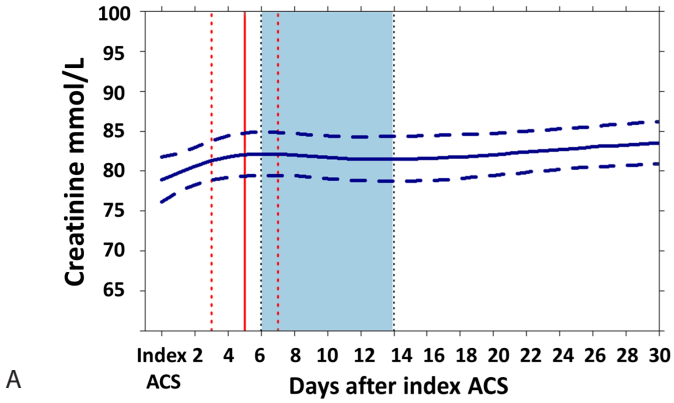
TABLE 1 **Baseline characteristics of the parent cohort and case-cohort set.**

Characteristics	All patients	Case-cohort		
		Non-cases	Cases	p-value
Number of patients	844	142	45	
Presentation and initial treatment				
Age, years *	62.5 (54.3, 70.2)	62.6 (55.0, 70.9)	67.4 (57.1, 76.5)	0.07
Male sex, %	77.9	78.2	80.0	0.79
Admission diagnosis, %				0.46
STEMI	51.7	45.8	35.6	
NSTEMI	37.7	39.4	48.9	
UAP	10.6	14.8	15.6	
Culprit artery, %				
RCA	33.1	34.5	26.7	0.33
LM	2.5	3.5	2.2	1.00
LAD	31.9	33.8	31.1	0.74
LCX	16.5	12.0	20.0	0.17
CABG performed, %	94.4	93.7	89.0	0.33
PCI performed, %	86.3	82.6	87.2	0.49
CKmax, U/L *	513 (200, 1370)	449 (190, 1197)	389 (194, 1122)	0.78
Killip class, %				0.012
Class I		94	82	
Class II		4	16	
Class III		2	0	
Class IV		0	2	
Renal function on admission:				
Urea, mmol/L *		5.9 (5.0, 7.0)	6.8 (4.7, 7.9)	0.19
Creatinine, umol/L *		82 (69, 95)	87 (73, 93)	0.22
eGFRCr, mL/min/1.73m^2 *		83 (69, 98)	78 (71, 92)	0.21
KDOQI classificationa, %				0.16
eGFRCr ≥90		35	24	
eGFRCr 60–89		55	60	
eGFRCr 30–59		10	16	

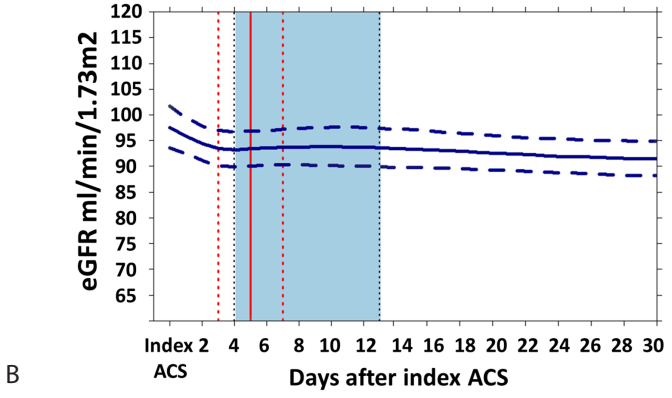
continued

Characteristics	All patients	Case-cohort		
		Non-cases	Cases	p-value
Medical history, %				
Diabetes mellitus	24	17	38	0.003
Hypertension	56	54	49	0.53
Dyslipidemia	49	51	44	0.46
Prior PCI	26	27	31	0.59
Prior CABG	10	9	24	0.004
Prior MI	27	30	31	0.92
Heart failure	2	3	9	0.097
Valvular heart disease	2	1	9	0.031
Prior CVA/TIA	9	11	20	0.13
PAD	9	6	22	0.004
Medication at first blood sampling moment from 7th day after index ACS, %				
Aspirin	95	93	100	0.20
P2Y12 inhibitor	95	90	97	0.46
Vitamin K antagonist	7	8	9.7	0.72
Statins	96	96	97	1.00
Beta-blocker	90	85	94	0.37
ACE inhibitor or ARB	84	84	90	0.57

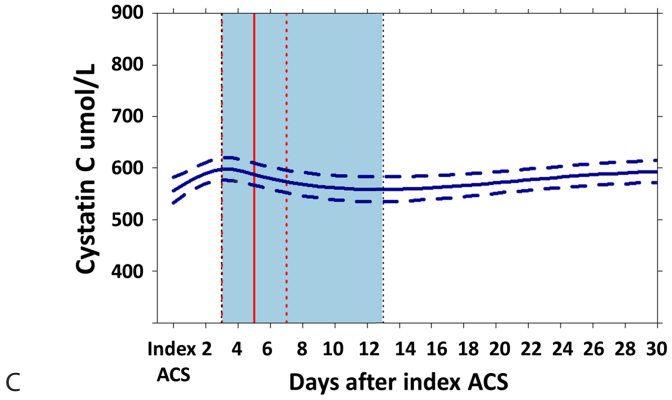
ACE: angiotensin converting enzyme; ARB: angiotensin II receptor blocker; CABG: coronary artery bypass grafting; CKmax: maximum creatine kinase during the index admission; LAD: left anterior descending artery; LCX: left circumflex artery; LM: left main coronary artery; NSTEMI: non-ST-elevation myocardial infarction; PCI: percutaneous coronary intervention; RCA: right coronary artery; STEMI: ST-elevation myocardial infarction; SD: standard deviation; Troponinmax: maximum troponin value during the index admission; UAP: unstable angina pectoris. * median (IQR)



A



B



C

FIGURE 2 Average evolutions of renal markers during washout phase. Follow-up time starting from admission is displayed on the x-axis. Biomarker levels are displayed on the y-axis. The solid red line depicts the median discharge day from hospital with corresponding interquartile range (dashed red lines). The left black dashed line displays time of the highest peak of plasma creatinine and CysC and the lowest peak of $eGFR_{Cr}$, and the right black dashed line displays the time moments of biomarker stabilization. The light blue area (between the two black dashed lines) represents the time period from the peaks/nadirs to stabilization. **A**. plasma creatinine (umol/L); **B**. $eGFR_{Cr}$ (ml/min/1.73m^2); **C**. plasma CysC (μg/ml).

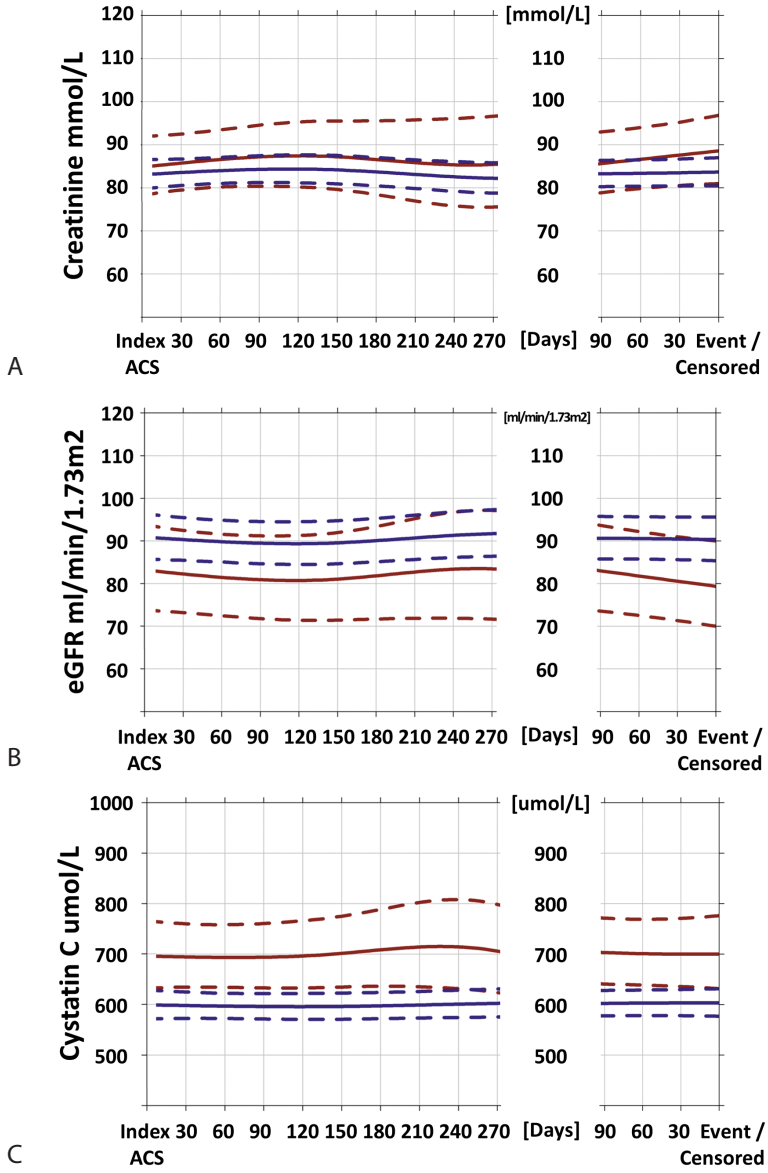


FIGURE 3 Average evolutions of renal markers during the year preceding death or recurrence of ACS in patients who reached the study endpoint (study endpoint cases) and last sample moment in patients who remained endpoint-free (non-endpoint patients). The solid red line depicts the average evolutions of renal function parameters in cases, and the solid blue line depicts the average evolutions in non-cases. The dashed lines represent the 95% confidence interval. Biomarker levels are displayed on the y-axis. X-axis: days from discharge or day 7 after index ACS until event (in study endpoint cases) or last sample moment (in non-endpoint patients). **A**. plasma creatinine (umol/L); **B**. eGFRCr (ml/min/1.73m^2); **C**. plasma CysC (µg/ml).

Predictive value of serially assessed renal markers during the year preceding death or the recurrence of ACS

No clear associations were found between serially assessed plasma creatinine or $eGFR_{Cr}$ and the study endpoints (Table 2). Conversely, serially measured CysC levels were positively associated with the endpoint (HR [95%CI]: per 1SD increase of ^{2}logCysC: 1.79 [1.21–2.63], p=0.006). After controlling for the GRACE risk score, CysC level remained a significant predictor (adjusted HR [95%CI]: 1.63 [1.01–2.66], p=0.043).

In the sensitivity analysis, CysC level measured serially >7 days after the index ACS was slightly weaker, but also a significant predictor (1.68 [1.13–2.46], p=0.009). After adjustment for the GRACE risk score, the risk estimates remained materially the same (adjusted HR [95%CI]: 1.63 [1.01–2.57], p=0.045) (Table S2). None of the rates of change of the renal biomarkers was associated with the endpoint (Table 2, and Table S2).

TABLE 2 Hazard ratios for the primary endpoint in relation to serially assessed marker levels >30 days after index ACS.

	Geometric mean**			Levels[a]		Instantaneous Slope[b]	
	Mean - 1SD	Mean	Mean + 1SD	HR (95%CI)	p-value	HR (95%CI)	p-value
Creatinine	67	84	105				
crude model				1.28 (0.84–1.97)	0.28	1.00 (0.53–1.85)	0.98
+ GRACE risk score [#,*]				1.12 (0.73–1.76)	0.61	1.00 (0.53–1.89)	0.99
eGFR	64	88	120				
crude model				1.52 (0.97–2.37)	0.06	1.00 (0.53–1.86)	1.00
+ GRACE risk score [#,*]				1.32 (0.85–2.10)	0.20	1.02 (0.56–1.87)	0.93
CysC	473.1	613.1	794.6				
crude model				1.79 (1.21–2.63)	0.006	0.99 (0.53–1.90)	0.98
+ GRACE risk score[#,*]				1.63 (1.01–2.66)	0.043	0.99 (0.53–1.83)	0.99

[a] Hazard ratios (HRs) and 95% confidence interval (CI) are given per 1SD increase (creatinine and cystatin C), and 1SD decrease (eGFR) on the 2-log scale at any time point after 30 days after index ACS.
[b] HRs (95%CI) are given per 0.1SD increase in the slope (creatinine and cysC), and 0.1SD decrease ($eGFR_{Cr}$) on the 2-log scale at any time point after 30 days after index ACS.
[#] longitudinal model adjusted for GRACE risk score, sex, diabetes, history of coronary artery bypass surgery, history of valvular heart disease, history of stroke, history of peripheral arterial disease.
* survival model adjusted for GRACE risk score.
** Geometric mean ± 1 standard deviation (SD) of the patient-specific biomarker values after 30 days (presented on the linear scale).

DISCUSSION

In this prospective multicenter study, we sought to describe the trajectories of renal function, and their impact on 1-year cardiac outcome in patients with ACS. We found that plasma CysC levels predict mortality or recurrence of ACS within the first year independently of the GRACE risk score. We also found that CysC levels are the earliest indicators of deterioration of renal function during index ACS. Importantly, we saw that renal function usually does not stabilize during hospitalization, but on average two weeks after index ACS. Altogether, these findings underscore the importance and complexity of renal dysfunction in ACS, and carry implications for the monitoring of renal function in these patients.

The majority of studies in patients with ACS have focused on prognostic value of creatinine levels or estimated GFR assessed at one point in time. However, the prognostic value of serial renal assessments, including CysC levels, is less clear and has mainly been investigated in patients with heart failure.[26] Although some authors[27] have speculated that assessment of renal function should be repeated after hospital discharge in patients with ACS, no study has examined evolution of renal function both during the washout phase early after ACS and during 1-year follow-up. It is here that our study further extends existing evidence.

Our findings support the incremental value of CysC levels for risk assessment by means of the GRACE score. Based on our findings, it seems reasonable to measure CysC levels in the time period after hospital discharge in patients for whom a more complete risk assessment is required. Comparable studies on repeated measurements are scarce. Akerblom et al. assessed whether repeatedly measured CysC levels (at baseline, discharge, and the mean value of both measurements) carry predictive value in 4295 patients with ACS and similar baseline creatinine levels as those in our study.[28] They reported that serial CysC assessment did not improve risk prediction. However, our results were obtained using a different approach. Contrary to Akerblom et al., we examined long-term temporal evolution of renal markers, specifically by using repeated measurements up to 1 year after hospital discharge to estimate the CysC trajectories in each patient. We then jointly modeled these renal trajectories with time-to-event analysis. This joint modeling approach carries several advantages. It enabled us to investigate the association with adverse events in a less biased way.[29] It also allowed us to examine the associations between the rates of change of different renal function parameters and adverse events. The latter analyses suggested that although CysC levels contribute to a patient's clinical risk, their rates of change do not. This is supported by Shlipak et al., who also could not demonstrate a significant association between change in creatinine (delta-creatinine ≥0.3 mg/dl) and outcomes in patients with stable coronary artery disease (CAD) in the Heart and Estrogen/Progestin Replacement Study

(HERS).[30] Thus, it appears that rate of change of renal function is only relevant for clinical risk in patients with CAD and systolic dysfunction, or with heart failure.[26,27,31]

Although we observed a slight deterioration of creatinine-based estimates prior to the incident endpoint, we could not confirm their predictive value as found previously.[1,2] This may be explained by the relatively low prevalence of patients with more severe renal dysfunction in our study. In fact, only 11% of our patients had moderate renal impairment ($eGFR_{Cr}$ 30–59) and there were no patients with $eGFR_{Cr}$ <30 due to the exclusion criteria. However, it appears that CysC levels were still able to detect these subtle differences, which may be of particular interest for patients with mild eGFR reduction ($eGFR_{Cr}$ 60–89), as was the case in 56% of patients included in the study. Although such mild renal dysfunction usually does not require medical attention, accurate monitoring of these subtle differences by cysC may carry potential for improving risk stratification of these patients.

Study limitations

Several aspects of our study warrant consideration. First, the MDRD equation, although validated in patients with ACS, has limitations due to the non-renal factors that influence creatinine measures. Nevertheless, we chose MDRD because it is the most widely utilized $eGFR_{Cr}$ equation, and thus enables comparisons with existing studies. Second, patients were excluded in case of $eGFR_{Cr}$ <30, which limits generalizability of our results to the ACS population at large. Yet we were able to demonstrate, even in this ACS population with a lesser degree of renal impairment, that renal dysfunction quantified by plasma CysC is associated with cardiovascular events. Third, despite controlling analyses for GRACE risk score – a risk model recommended in international guidelines - residual confounding may still be present.

CONCLUSION

During hospitalization for ACS, plasma CysC levels indicate deterioration of renal function earlier than creatinine or $eGFR_{Cr}$. Renal function usually does not stabilize during hospitalization, but on average two weeks after ACS. In patients with normal to moderately-impaired renal function, CysC levels predict mortality or recurrence of ACS within the first year independently of GRACE risk score.

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SUPPLEMENTARY INFORMATION

TABLE S1. Inclusion and exclusion criteria

Inclusion: a patient must meet all criteria	
1	Age ≥40 years
2	Complaints of typical ischemic chest pain, lasting 10 minutes or more within the preceding 24 hours prior to presentation
3a	ECG: (non-)persistent ST segment elevation >1.0 mm in two or more contiguous leads, or dynamic ST segment depression >1.0 mm in two or more contiguous leads, *OR*
3b	Biochemical evidence of myocardial injury: CK-MB or (high-sensitivity) Troponin I or (high-sensitivity) Troponin T elevation according to the applicable ESC guidelines of non ST-elevation acute coronary syndromes
4	Presence of at least 1 of the following risk factors: age ≥75 years, diabetes, prior cardiovascular disease, prior cerebrovascular disease and prior peripheral arterial disease. In addition, other risk factors mentioned below can be considered as well, but each only counts as half a risk factor, i.e., two of these are required for inclusion: age ≥65 years in men, age ≥70 years in females, hypertension, hypercholesterolemia, current smoking, or microalbuminuria†, positive family history of coronary artery disease‡
5	Written informed consent

continued

Exclusion: a patient cannot be included in case of any of the criteria below

1	Myocardial ischemia precipitated by a condition other than atherosclerotic coronary artery disease
2	Left ventricular ejection fraction <30%, or end-stage congestive heart failure (NYHA class III or IV)
3	Renal dialysis, or severe chronic kidney disease with measured or calculated GFR_{Cr} (Cockroft-Gault or MDRD formula) of <30 ml/min/1.73 m^2
4	Co-existent condition with life-expectancy <1 year or otherwise not expected to complete follow-up

GFR_{Cr}: glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; NYHA: New York Heart Association classification
† defined as >2.5-25 mg albumin/mmol creatinine for men and >3.5-35 mg for women, or >20-200 mg/l urinary albumin concentration in a single urine sample
‡ angina pectoris, myocardial infarction, or sudden abrupt death without obvious cause, before the age of 55 in a first-degree blood relative

TABLE S2 Hazard ratios for the primary endpoint in relation to biomarker levels >7 after ACS.

	Geometric mean**			Levels[a]		Instantaneous slope[b]	
	Mean - 1SD	**Mean**	**Mean + 1SD**	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**
Creatinine	67	84	105				
crude model				1.40 (0.94–1.98)	0.09	1.00 (0.53–1.85)	0.98
+ GRACE risk score[#,*]				1.29 (0.86–1.94)	0.61	1.01 (0.54–1.90)	0.98
$eGFR_{Cr}$	64	89	122				
crude model				1.42 (0.97–2.06)	0.08	1.01 (0.54–1.87)	0.93
+ GRACE risk score[#,*]				1.25 (0.82–1.89)	0.30	1.01 (0.53–1.91)	0.97
CysC	466.9	608.9	794.0				
crude model				1.68 (1.13–2.46)	0.009	1.00 (0.54–1.78)	0.98
+ GRACE risk score[#,*]				1.63 (1.01–2.57)	0.045	0.99 (0.53–1.82)	0.95

[a] Hazard ratios (HRs) and 95% confidence interval (CI) are given per 1SD increase (creatinine and cystatin C), and 1SD decrease ($eGFR_{Cr}$) on the 2-log scale at any time point after 7 days after index ACS.
[b] HRs (95%) CI are given per 0.1SD increase in the slope (creatinine and CysC), and 0.1SD decrease ($eGFR_{Cr}$) on the 2-log scale at any time point after 7 days after index ACS.
[#] longitudinal model adjusted for GRACE risk score, sex, diabetes, history of coronary artery bypass surgery, history of valvular heart disease, history of stroke, history of peripheral arterial disease.
* survival model adjusted for GRACE risk score.
** Geometric mean ± 1 standard deviation (SD) of the patient-specific biomarker values after 30 days (presented on the linear scale).

CHAPTER 10

Plasma Cystatin C and Neutrophil Gelatinase-Associated Lipocalin in Relation to Coronary Atherosclerosis on Intravascular Ultrasound and Cardiovascular Outcome Impact of Kidney Function The AtheroRemo-IVUS Study

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ABSTRACT

Background

We investigated whether plasma cystatin C (CysC) and neutrophil gelatinase-associated lipocalin (NGAL) are associated with intravascular ultrasound (IVUS)-derived characteristics of coronary atherosclerosis and 1-year adverse coronary events in patients with normal and mildly-to-moderately impaired kidney function.

Methods

Between 2008-2011, virtual histology (VH)-IVUS of a non-culprit coronary artery was performed in 581 patients undergoing coronary angiography. Creatinine, CysC and NGAL were measured in pre-procedural blood samples. Presence of VH-IVUS-derived thin-cap fibroatheroma (TCFA) lesions, lesions with plaque burden (PB)≥70% and lesions with minimal luminal area (MLA)≤4 mm^2 was assessed. Major adverse coronary events (MACE) comprised the composite of all-cause mortality, acute coronary syndrome, or unplanned coronary revascularization. Analyses were stratified using $eGFR_{Cr}$ of 90 ml/min/1.73m^2 as the cut-off.

Results

In patients with normal kidney function, those with higher CysC levels had fewer lesions with PB≥70% and fewer VH-TCFA lesions (adjusted odds ratios(ORs) and 95% confidence intervals(CIs): 0.46 [0.30-0.69] and 0.59 [0.44-0.83], respectively, per standard deviation(SD) ln[ng/mL] CysC). Those with higher NGAL levels also had fewer lesions with PB≥70% (adjusted OR [95%CI]: 0.49 [0.29-0.82]) In patients with impaired kidneys, no differences in high-risk lesions were observed for CysC or NGAL. However, those with higher CysC had higher risk of MACE (hazard ratio(HR): 1.4, 95%CI [1.03–1.92]). This was not the case in patients with normal kidney function. NGAL did not influence risk of MACE.

Conclusions

Mild-to-moderate kidney dysfunction modifies the relationship between CysC and high-risk coronary lesions. This has not been established before, and offers an explanation for the difference in findings between experimental and epidemiologic studies.

INTRODUCTION

Kidney impairment, as assessed by creatinine-based equations of glomerular filtration rate ($eGFR_{Cr}$), is associated with cardiovascular disease independently of established cardiovascular risk factors.[1] In persons with mild kidney dysfunction ($eGFR_{Cr}$ in the range of 60-89 ml/min/1.73m^2), cystatin C (CysC) may outperform $eGFR_{Cr}$ as a predictor of adverse outcome. This is illustrated by the fact that CysC displays a linear association with mortality in patients with such mild GFR reduction, while $eGFR_{Cr}$ has a J-shaped association with mortality, and risk only starts to rise when $eGFR_{Cr}$ falls beneath 60 ml/min/1.73m^2.[2,3] Although some studies have shown linear associations of $eGFR_{Cr}$ with adverse outcome, these associations were linear only in particular ranges of $eGFR_{Cr}$ (specifically, $eGFR_{Cr}$ above 60).[4]

CysC is a cysteine protease inhibitor produced by most nucleated cells, and can be detected in serum or plasma.[5] In in-vitro and animal experiments, a reduction of CysC correlated with increased activity of cysteine proteases cathepsins K and S, which led to breakdown of the elastic lamina in the blood vessel wall.[6] Altered CysC expression has been identified in diseases which progress by extracellular proteolysis, such as atherosclerosis and aortic aneurysms, and metastasis.[7,8] These experiments, pointing towards a favourable role for CysC, do not concur with the positive associations of CysC with adverse outcomes found in epidemiological studies. Studies on the in-vivo association between plasma CysC and coronary atherosclerosis may provide further insight into this discrepancy, but have not yet been performed.

Neutrophil gelatinase-associated lipocalin (NGAL) is a clinically relevant biomarker in acute kidney injury[9] due to its marked increase in plasma and urine after tubulointerstitial kidney damage.[10] Recently, overexpression of plasma NGAL has been found in coronary plaques, where NGAL inhibits elimination of matrix metalloproteinase–9 (MMP-9).[11,12] MMP-9 is involved in extracellular matrix degradation, herewith increasing the risk of plaque rupture.[13] NGAL and NGAL/MMP-9 complex have been shown to predict major adverse cardiovascular events in epidemiological studies.[14,15]

In spite of the above-described associations that have been demonstrated between CysC, NGAL and adverse cardiac events, the presence and shape of a relationship between plasma CysC, NGAL, and coronary atherosclerosis have not yet been investigated in-vivo. To the best of our knowledge, we are the first to perform such an investigation, and to herewith provide a link between fundamental experiments and epidemiological studies. Specifically, our study aimed to investigate whether plasma CysC and NGAL are associated with IVUS-derived characteristics of in-vivo coronary atherosclerosis and 1-year adverse coronary events in patients with normal and mildly-to-moderately impaired kidney function.

MATERIALS AND METHODS

Study population

We have previously described the design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis - Intravascular Ultrasound (ATHEROREMO-IVUS).[16] In this study, we included 581 patients undergoing diagnostic coronary angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS) or stable angina pectoris (SAP) between 2008 and 2011 in the Erasmus MC, Rotterdam, the Netherlands. Following coronary angiography, intravascular ultrasound (IVUS) of a non-culprit coronary artery was performed. The human research ethics committee of Erasmus MC, Rotterdam, the Netherlands has approved this study. All included patients have signed informed consent, and the study protocol conformed to the Declaration of Helsinki. This study is registered in ClinicalTrials.gov (number: NCT01789411).

Kidney function assessment

Estimated Glomerular Filtration Rate ($eGFR_{Cr}$) was assessed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[17] Patients were categorized according to eGFR by using the modified definition from the National Kidney Foundation – Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines[18]: normal (GFR≥90 ml/min/1.73m^2), mild (GFR 60-89 ml/min/1.73m^2), moderate (GFR 30-59 ml/min/1.73m^2), and severe (GFR 15-29 ml/min/1.73m^2) kidney dysfunction, and kidney failure (GFR<15 ml/min/1.73m^2). No patients with kidney failure were present in this study, and only one patient had $eGFR_{Cr}$ <30 ml/min/1.73m^2. The latter was excluded from further analyses. Patients were stratified into those with normal kidney function and those with mildly-to-moderately impaired kidney function, using an $eGFR_{Cr}$ of 90 ml/min/1.73m^2 as the cut-off value.

Biomarkers

Arterial blood was taken before the procedure and stored at -80 °C within two hours. Samples were available in 570 patients. An immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Roche Cobas 8000 modular analyser platform was used in the Erasmus MC clinical laboratory to measure the level of C-reactive protein (CRP) in serum samples. The plasma EDTA samples were transported at a temperature of -80 °C to Myriad RBM, Austin, Texas,

USA, where cystatin C and NGAL concentrations were assessed by a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, Texas, USA). As a result of the batch-wise handling of the samples, with an update of the composition of the multiplex assay by the manufacturer in-between two batches, cystatin C was measured in the full cohort of 570 patients, and NGAL in a random subset of 473 patients. Both laboratories were blinded to clinical and imaging data.

Grayscale and radiofrequency intravascular ultrasound (IVUS)

The degree (plaque volume and plaque burden) and composition of the atherosclerotic plaque were assessed. Plaque volume was defined as the total volume of the external elastic membrane occupied by atheroma.[19] Plaque burden was defined as the plaque and media cross-sectional area divided by the external elastic membrane cross-sectional area and is presented as a percentage (Figure 1). A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least three consecutive frames.[16] The composition of the atherosclerotic plaque was characterized into fibrous, fibro-fatty, dense calcium and necrotic core.[20] Subsequently, three types of VH-IVUS high-risk lesions were identified: 1. thin-cap fibroatheroma (TCFA) lesion: a lesion with the presence of >10% confluent necrotic core in direct contact with the lumen; 2. lesion with a plaque burden of ≥70%; 3. lesion with a minimal luminal area (MLA) of ≤4.0mm^2.[21]

Follow-up

Clinical follow-up started at inclusion and lasted one year. The primary clinical endpoint − MACE − was the composite of all-cause mortality, ACS, or unplanned coronary revascularization. ACS was defined as the clinical diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI, or unstable angina pectoris using the guidelines of the European Society of Cardiology.[22,23] Unplanned coronary revascularizations were defined as unplanned coronary artery bypass grafting or repeat percutaneous coronary intervention. The secondary endpoint was the composite of all-cause mortality or ACS. The endpoints were adjudicated by a clinical event committee blinded for biomarker and IVUS data.

Statistical analysis

The Kolmogorov-Smirnov test was used to test distributions of continuous variables for normality. CysC and CRP were not normally distributed and were ln-transformed for further analyses. Categorical variables are presented as numbers

and percentages. Continuous variables that were normally distributed are presented as mean±standard deviation (SD); non-normally distributed continuous variables are presented as median and interquartile range (IQR). For reasons of uniformity, all biomarkers are presented as median (IQR).

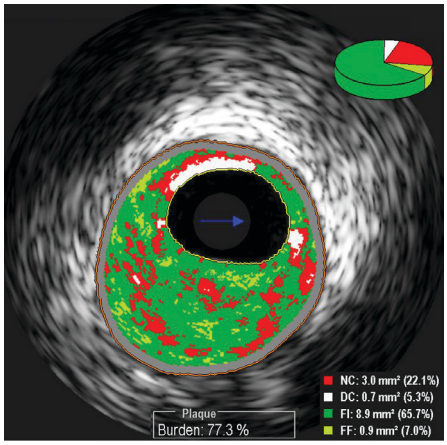
We examined the associations of plasma CysC and NGAL levels with plaque burden, plaque volume, and the presence of high-risk coronary lesions. Plaque volume was normalized for the imaged segment length. We used linear regression and logistic regression analyses with continuous ln-transformed CysC and NGAL concentrations consecutively as independent variables. To assess the effect of kidney function, we included interaction terms (ln-transformed CysC or NGAL, respectively, with dichotomized $eGFR_{Cr}$ (above or below 90 ml/min/1.73m^2)) into the logistic regression models. Subsequently, we stratified all analyses on $eGFR_{Cr}$ of 90 ml/min/1.73m^2. To test whether effect estimates differed between patients with ACS and patients with SAP, Z-tests for heterogeneity were performed.

Cox proportional hazards regression analyses were performed to evaluate the associations between CysC and NGAL and the clinical study endpoints.

Age, gender, indication for coronary angiography, diabetes mellitus, hypertension, and CRP concentration were considered as potential confounders , and were therefore entered into the multivariable linear and logistic regression models. Multivariable adjustment of Cox proportional hazards models was constrained due to the number of clinical endpoints, and was therefore performed in two steps. For MACE, in the first step the adjustment included age, gender, and indication for angiography; in the second step, diabetes mellitus, hypertension and CRP were added.

Finally, we determined the cut-off values of CysC and NGAL that carry the optimal discriminative ability with respect to presence of high-risk coronary lesions and occurrence of MACE. For this purpose, we drew receiver operating characteristic (ROC) curves and calculated the Youden index (highest sum of sensitivity and specificity -1).[24] We considered only statistically significant associations.

All data were analysed with SPSS software (SPSS 20.0; IBM Corp., Armonk, NY). All statistical tests were two tailed, and p values <0.05 were considered statistically significant.



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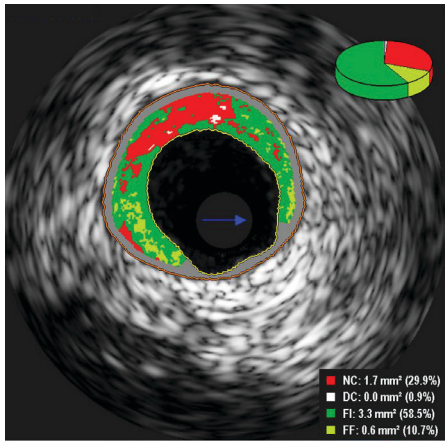

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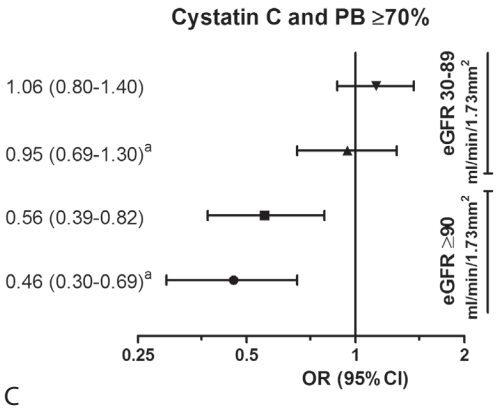

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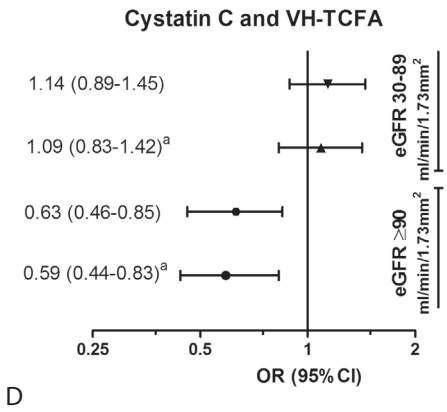

D

FIGURE 1 Plasma cystatin C and presence of VH-IVUS high-risk coronary lesions. A. Lesion with plaque burden (PB) ≥70%. Plaque burden is defined as plaque and media cross-sectional area (i.e., area between yellow contour and red contour) divided by external elastic membrane cross-sectional area (contoured in red); **B.** VH-IVUS derived thin-cap fibroatheroma lesion (VH-TCFA), defined as a lesion (i.e., plaque with a plaque burden >40%) with presence of confluent necrotic core >10% in direct contact with the lumen in at least three frames; **C.** Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) for lesions with PB ≥70%. **D.** Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) for VH-TCFA lesions.
FI, fibrous; FF, fibro-fatty; NC, necrotic core, DC, dense calcium.
[a] adjusted for age, gender. diabetes, hypertension, indication for angiography, C-reactive protein.

RESULTS

Baseline characteristics

Mean age was 61.6±11.4 years, 75.7% were men, 54.6% had ACS, and 45.4% had SAP (Table 1). The imaged coronary segment had a median length of 44.3(33.8-55.4)mm. A total of 239 (41.5%) patients had at least one TCFA lesion, 120 (21.0%) had lesions with PB ≥70%, and 175 (30.7%) had lesions with MLA ≤4 mm^2. Median $eGFR_{Cr}$ was 90 (77-98) ml/min/1.73mm^2 in the full cohort with similar values in the subset of ACS patients (91[78-100] ml/min/1.73mm^2) and SAP patients (89 [77-97] ml/min/1.73mm^2). A total of 291 (51.8%) patients had normal kidney function and 271 (48.2%) patients had mild-to-moderate kidney dysfunction. ACS patients exhibited significantly higher NGAL levels compared to patients with SAP, regardless of kidney function, whereas plasma CysC levels were similar in both $eGFR_{Cr}$ groups (Table 1).

TABLE 1 Baseline characteristics.

Variable	Total (n=570)	ACS patients (n= 309)	SAP patients (n=261)
Patient characteristics			
Age, years (mean ± SD)	61.5±11.4	59.7±11.9	63.6±10.3
Men, n (%)	430 (75.4)	227 (73.5)	203 (77.8)
Diabetes Mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Hypercholesterolemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Positive family history, n (%)	293 (51.5)	140 (45.5)	153 (58.6)
Previous MI, n (%)	184 (32.3)	80 (25.9)	104 (39.8)
Previous PCI, n (%)	185 (32.5)	57 (18.4)	128 (49.0)
Previous CABG, n (%)	18 (3.2)	7 (2.3)	11 (4.2)
Previous stroke, n (%)	23 (4.0)	10 (3.2)	13 (5.0)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
Indication for coronary angiography			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100.0)	0 (0.0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0.0)
Unstable angina pectoris, n (%)	150 (26.3)	150 (48.5)	0 (0.0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0.0)	261 (100.0)

continued

Variable	Total (n=570)	ACS patients (n= 309)	SAP patients (n=261)
Coronary artery disease [a]			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.5)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
IVUS characteristics			
Segment length (mm), median (IQR)	44.2 (33.7-55.4)	43.9 (32.9-54.1)	44.8 (34.2-57.2)
Plaque burden (%), median (IQR)	39.2 (29.9-46.4)	37.2 (28.0-45.5)	40.1 (31.8-47.7)
Presence of VH-TCFA, n (%)	239 (41.9)	140 (45.5)	99 (37.9)
Presence of PB ≥70%, n (%)	120 (21.0)	56 (18.1)	64 (24.5)
Presence of MLA ≤4 mm^2	175 (30.7)	87 (28.2)	88 (33.7)
Renal function			
eGFR (ml/min/1.73m^2) median (IQR) [b,c]	90 (77-98)	91 (78-100)	89 (77-97)
KDOQI classification[a], n (%)			
GFR ≥90 ml/min/1.73m^2	291 (51.8)	165 (54.3)	126 (48.8)
GFR 60-89 ml/min/1.73m^2	231(41.1)	115 (37.8)	116 (45.0)
GFR 30-59 ml/min/1.73m^2	39 (6.9)	23 (7.6)	16 (6.2)
GFR <30 ml/min/1.73m^2	1 (0.1)	1 (0.3)	0 (0.0)
Serum biomarkers			
NGAL (ng/mL) median (IQR) [d]	197.0 (143.0-254.0)	204.0 (148.2-274.5)	177.0 (141.5-239.0)
$eGFR_{Cr}$ ≥90 ml/min/1.73m^2 [e]	183.0 (143.0-227.0)	193.0 (143.0-243.0)	174.0 (125.0-223.0)
$eGFR_{Cr}$ 30-89 ml/min/1.73m^2 [e]	216.0 (148.0-293.2)	228.5 (149.0-307.0)	197.0 (143.5-257.7)
Cystatin C (ng/ml) median (IQR)	796.0 (691.0-923.0)	791.0 (674.5-915.5)	802.0 (712.5-935.5)
$eGFR_{Cr}$ ≥90 ml/min/1.73m^2	732. 0 (644.0-834.0)	729.0 (637.5-841.5	734.5 (650.7-822.5)
$eGFR_{Cr}$ 30-89 ml/min/1.73m^2	872.0 (775.7-1032.5)	863.0 (745.0-1040.0)	879.0 (781.0-1030.0)
Creatinine (umol/l), median (IQR) [c]	77 (66-86)	77 (65-877)	76 (67-86)
C-reactive protein (mg/l), median (IQR)	2.1 (0.8-5.3)	2.8 (1.1-6.9)	1.4 (0.6-3.1)

ACS, acute coronary syndrome; SAP, stable angina pectoris; CABG, coronary artery bypass grafting; MI, myocardial infarction; PCI, percutaneous coronary intervention; CKD, chronic kidney disease; NGAL, neutrophil gelatinase-associated lipocalin; PB, plaque burden; MLA, minimal luminal area. [a] significant stenosis was defined as a stenosis >50% of the vessel diameter by visual assessment on the coronary angiogram. [b] estimated Glomerular Filtration Rate (eGFRCr) using CKD-EPI equation: $GFR = 141 \times min(Scr/\kappa, 1)^{\alpha} \times max(Scr$

/κ, 1)-1.209 × 0.993Age × 1.018 [if female] × 1.159 [if black] where: Scr is serum creatinine in mg/dL, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr /κ or 1, and max indicates the maximum of Scr /κ or 1. [c] Creatinine available in 99%, Total n=562, ACS n=304, SAP n=258; [d] Measurable in sample of total n=473, ACS n=257, SAP n=216. [e] A statistically significant difference in plasma NGAL levels between ACS and SAP patients (for total population, p=0.002; if $eGFR_{Cr}$ ≥90 ml/min/1.73 m^2, p=0.01; if $eGFR_{Cr}$ 30-89 ml/min/1.73 m^2, p=0.03).

Cystatin C, NGAL and degree of atherosclerosis on grayscale IVUS

Numbers of lesions with plaque burden (PB) ≥70% and minimal luminal area (MLA) ≤4mm^2 according to categories of kidney function are depicted in Figure S3. Significant interactions were found between CysC and $eGFR_{Cr}$ in crude (p=0.007) and multivariable (p=0.010) models predicting lesions with PB ≥70%. In patients with normal kidney function, those with higher CysC had lower risk of lesions with PB ≥70% (per SD increase in ln-transformed CysC: OR[95%CI]: 0.56 [0.39-0.82], p=0.002) (Table 2, Figure 1, Figure S1). After multivariable adjustment including CRP levels, risk remained significantly lower (adjusted OR[95%CI]: 0.46 [0.30-0.69], p<0.001). A CysC level of 773.0 ng/ml was the optimal cut-off value to identify patients who did not have lesions with PB≥70% (CysC ≥773.0 ng/ml) (Figure S4). Conversely, in patients with mild-to-moderate kidney dysfunction risk did not differ significantly according to CysC levels (adjusted OR[95%CI]:0.95 [0.69-1.30], p=0.75). Risk of lesions with PB ≥70% displayed a similar pattern in patients with higher NGAL (Table 2). In patients with normal kidney function, an NGAL level of 180.0 ng/ml was the optimal cut-off value to identify patients without lesions with PB ≥70% (NGAL ≥180.0 ng/ml) (Figure S5). Risk of lesions with MLA ≤4mm^2 was not different for patients with higher CysC or NGAL (Table 2).

Overall, no differences could be demonstrated between CysC and NGAL in either plaque burden or normalized plaque volume of the entirely imaged segment (Table 3 and Table S2). Nevertheless, CysC showed a tendency towards lower normalized segment plaque volume (per SD increase in ln-transformed CysC: β [95%CI]: -0.43 [-1.02-0.16], p=0.16) in patients with normal kidney function; whereas no differences were observed in patients with mild-to-moderate kidney dysfunction.

There was no heterogeneity between ACS and SAP patients regarding the differences in IVUS grayscale parameters according to CysC or NGAL levels.

TABLE 2 Plasma cystatin C, NGAL and presence of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB) ≥70% and lesions with minimal luminal area (MLA) ≤4mm² stratified according to kidney function.

	Unadjusted model		Multivariable model	
	OR (95% CI)	p-value	OR (95% CI)	p-value
$eGFR_{Cr}$ ≥90 ml/min/1.73m²				
VH-TCFA				
Cystatin C [a]	0.63 (0.46-0.85)	0.002	0.59 (0.44-0.83)	0.002
NGAL [b]	0.77 (0.57-1.04)	0.090	0.72 (0.52-0.98)	0.040
Plaque Burden ≥70%				
Cystatin C [a]	0.56 (0.39-0.82)	0.002	0.46 (0.30-0.69)	<0.001
NGAL [b]	0.56 (0.35-0.89)	0.015	0.49 (0.29-0.82)	0.007
MLA ≤4mm²				
Cystatin C [a]	0.97 (0.72-1.32)	0.88	0.92 (0.67-1.25)	0.59
NGAL [b]	1.03 (0.77-1.37)	0.84	1.07 (0.79-1.45)	0.67
$eGFR_{Cr}$ 30-89 ml/min/1.73m²				
VH-TCFA				
Cystatin C [a]	1.14 (0.89-1.45)	0.30	1.09 (0.83-1.42)	0.55
NGAL [b]	1.01 (0.78-1.29)	0.97	0.96 (0.74-1.24)	0.74
Plaque Burden ≥70%				
Cystatin C [a]	1.06 (0.80-1.40)	0.68	0.95 (0.69-1.30)	0.75
NGAL [b]	1.20 (0.88-1.63)	0.25	1.21 (0.87-1.67)	0.25
MLA ≤4 mm²				
Cystatin C [a]	0.84 (0.64-1.10)	0.19	0.73 (0.53-0.99)	0.042
NGAL [b]	0.98 (0.74-1.29)	0.90	1.05 (0.78-1.41)	0.75

[a] Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI); [b] Odds ratio (OR) per standard deviation increase in NGAL with 95% confidence interval (CI); Multivariable model: adjusted for age, gender, diabetes mellitus, hypertension, indication for angiography, C-reactive protein.

Cystatin C, NGAL and composition of atherosclerosis on radiofrequency VH-IVUS

Absolute numbers of thin-cap fibroatheroma lesions (VH-TCFAs) according to categories of kidney function are depicted in Figure S3. Significant interactions were found between CysC and $eGFR_{Cr}$ in crude (p=0.002) and multivariable (p=0.003) models predicting VH-TCFAs. In patients with normal kidney function, those with

higher CysC levels had lower risk of VH-TCFA lesions (per SD increase in ln-transformed CysC: OR[95%CI]: 0.63 [0.46-0.85], p=0.002) (Table 2, Figure 1, Figure S1). After multivariable adjustment including CRP levels, risk remained significantly lower (adjusted OR[95%CI]: 0.59 [0.44-0.83], p=0.002). CysC of 678.5 ng/ml was the optimal cut-off value to identify patients without VH-TCFA lesions (CysC ≥678.5 ng/ml) (Figure S6). Conversely, in patients with mild-to-moderate kidney dysfunction, risk did not differ significantly according to CysC levels (adjusted OR[95%CI]: 1.09[0.83-1.42], p=0.55). The interaction between NGAL and $eGFR_{Cr}$ was not statistically significant. A tendency towards lower risk of VH-TCFA lesions was observed for higher NGAL, but only in patients with normal kidney function (Table 2). There was no heterogeneity between ACS and SAP patients regarding the difference in VH-TCFA lesions (CysC, p=0.29, NGAL, p=0.57) (Table S1). At the level of the entire segment, no differences were present in radiofrequency VH-tissue types between CysC or NGAL (Table 3 and Table S2).

TABLE 3 Plasma cystatin C, NGAL and segment characteristics (degree of atherosclerosis: plaque volume and plaque burden; composition of coronary atherosclerosis: 4 components) as determined by VH-IVUS stratified according to kidney function.

	Cystatin C [b]		NGAL [c]	
	β coefficient (95% CI)	p-value	β coefficient (95% CI)	p-value
$eGFR_{Cr}$ ≥90 ml/min/1.73m²				
Plaque burden [a]	-0.02 (-0.16 – 0.12)	0.77	-0.05 (-0.18 – 0.09)	0.50
Plaque volume [a]	-0.43 (-1.02 – 0.16)	0.16	-0.19 (-0.77 – 0.38)	0.51
FI (%)	0.52 (-1.11 – 2.15)	0.53	0.60 (-0.98 – 2.19)	0.45
FF (%) [a]	0.03 (-1.10 – 0.17)	0.65	0.12 (-0.02 – 0.25)	0.09
NC (%)	-0.65 (-1.84 – 0.53)	0.28	-0.85 (-2.00– 0.30)	0.15
DC (%) [a]	0.00 (-0.17 – 0.17)	0.99	-0.12 (-0.28 – 0.04)	0.15
$eGFR_{Cr}$ 30-89 ml/min/1.73m²				
Plaque burden [a]	0.00 (-0.11 – 0.12)	0.94	-0.03 (-0.15 – 0.09)	0.66
Plaque volume [a]	0.16 (-0.37 – 0.68)	0.55	-0.04 (-0.59 – 0.51)	0.89
FI (%)	-1.04 (-2.45 – 0.37)	0.15	0.60 (-0.89 – 2.09)	0.42
FF (%) [a]	-0.02 (-0.13 – 0.10)	0.76	-0.01 (-0.12 – 0.11)	0.92
NC (%)	0.44 (-0.47 – 1.35)	0.34	-0.27 (-1.23 – 0.68)	0.57
DC (%) [a]	0.11 (-0.04 – 0.25)	0.15	-0.06 (-0.21 – 0.09)	0.44

FI, fibrous; FF, fibro-fatty; NC, necrotic core, DC, dense calcium. [a] Square root transformed

[b] Unadjusted β coefficient per standard deviation increase in ln-transformed Cystatin C with 95% confidence interval (CI).
[c] Unadjusted β coefficient per standard deviation increase in NGAL with 95% confidence interval (CI).

Cystatin C, NGAL and 1-year MACE

Vital status was acquired for 569 (99.8%) patients. During the 1-year follow-up, 56 patients experienced the primary endpoint (MACE; Figure S3), and 30 patients endured the secondary composite endpoint of all-cause mortality or ACS. In the full cohort, patients with higher CysC had higher risk of MACE (per SD increase in ln-transformed CysC: HR[95% CI]:1.41[1.10-1.79], p=0.006) (Figure 2, Figure S2). After multivariable adjustment, the risk estimate lost statistical significance. For NGAL, significant differences in risk of MACE were not found (Figure 2, and Figure S2).

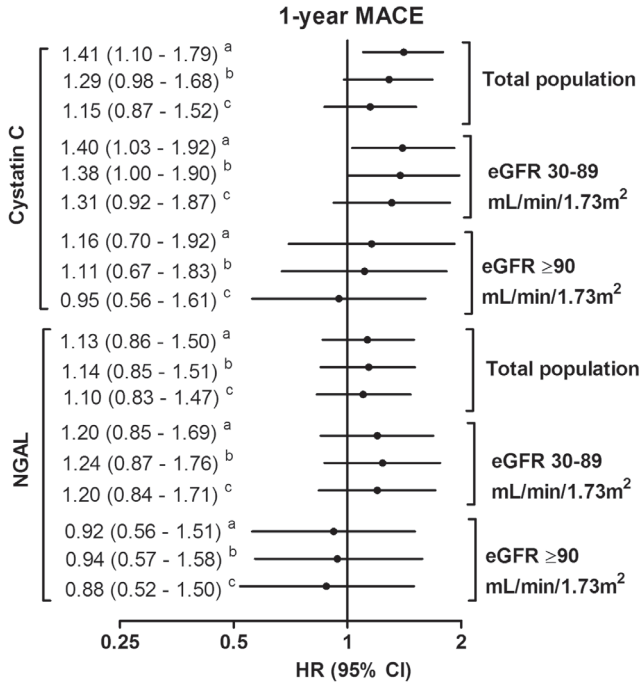

FIGURE 2 Plasma cystatin C, NGAL and occurrence of the 1-year MACE. MACE, major adverse coronary event; Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C and per standard deviation increase in NGAL with 95% confidence interval (CI). [a] unadjusted model; [b] adjusted for age, gender, indication for angiography; [c] adjusted for age, gender, indication for angiography, diabetes mellitus, hypertension, C-reactive protein; Multivariable adjustment was constrained by the limited number of clinical endpoint.

In patients with normal kidney function, those with higher CysC levels did not have higher risk of MACE (Figure 2, Figure S2). In patients with mild-to-moderate kidney dysfunction, those with higher CysC levels had higher risk of MACE in univariable analysis (HR[95%CI]:1.40[1.03-1.92], p=0.03) (Figure 2, Figure S2). In multivariable analysis, the HR lost statistical significance, but did not materially change (HR[95%CI]:1.31 [0.92-1.87], p=0.12).

Both in the total population and in patients with mild-to-moderate kidney dysfunction, a CysC of 849.0 ng/ml was the optimal cut-off value to identify patients who developed MACE (CysC ≥849.0 ng/ml) (Figure S7). Patterns of risk of the secondary endpoint (all-cause mortality and ACS) according to CysC and NGAL levels were similar to those of MACE (Table S3). Finally, stratification on the indication for angiography confirmed the risk patterns which were found in the full cohort (Table S4).

DISCUSSION

We found that in patients with normal kidney function, those with higher CysC levels had fewer high-risk coronary lesions (VH-TCFA and lesions with PB ≥70%), while risk of MACE was not different. Conversely, when kidney function was mildly-to-moderately impaired, no differences in high-risk lesions were observed, but those with higher CysC levels had higher risk of MACE. Therefore, with regard to prediction of cardiovascular risk, CysC appears to carry potential only when eGFR$_{Cr}$ is below 90 ml/min/1.73m^2. Furthermore, patients with higher NGAL levels had fewer lesions with PB≥70%, but only when they had normal kidney function. No differences in MACE were found for NGAL, and thus its use for cardiovascular risk prediction could not be substantiated. Altogether, our results on CysC suggest novel pathophysiological insights, because they offer an explanation for the difference in findings observed in experimental and epidemiologic studies so far, and imply that the association between CysC and cardiovascular disease may not be solely explained through its correlation with GFR.

Higher CysC levels have been associated with occurrence of cardiovascular events in various epidemiological studies.[25] Conversely, animal experiments suggest that higher CysC may be favourable. Atherosclerotic mice deficient in CysC display increased plaque size and macrophage content, increased elastic lamina degradation and accumulation of smooth muscle cells.[26,6] Studies in humans have also found reduced CysC in atherosclerotic and aneurysmatic aortic lesions.[7] Xu et al. have demonstrated that immune cells (CD8+ dendritic cells (DC) and macrophages), which are involved in atherosclerotic processes, are major contributors to

the circulating CysC pool.[27,28] However, besides a correlation with GFR, the mechanisms that may explain the link between CysC and cardiovascular disease are still unclear. Our study provides additional insights. We found that in patients with normal kidney function, those with higher CysC levels had fewer high-risk coronary lesions, and did not have higher risk of MACE. This is in accordance with a potential 'athero-protective' effect.

Conversely, in patients with mild-to-moderate kidney dysfunction, differences in high-risk lesions according to CysC level were not present. This could possibly be explained by the changes in CysC physiology that occur in impaired kidneys. When kidney function deteriorates, circulating plasma CysC increases and oxidative stress advances, both of which stimulate Cys to form homodimers.[28,29] When CysC forms homodimers, it cannot inhibit cysteine proteases, because the inhibitory region is hidden within the dimer interface. Thus, it may no longer be able to exhibit 'athero-protective' properties.[30] Although these hypotheses are compelling, additional clinical and experimental studies are necessary to further substantiate the effect modification by kidney function that we observed.

Our findings suggest that NGAL may act on coronary artery disease through a different mechanism than currently investigated. A potential lack of predictive precision due to a limited number of MACE may explain the difference between the current results and previous studies.[15,31] On the other hand, a recent meta-analysis that investigated NGAL as a predictor of cardiovascular disease concluded that strong evidence for independent predictive value of NGAL is still lacking.[32] Notably, we found higher plasma NGAL levels in ACS patients compared to SAP patients, independently of kidney function. This could possibly be explained by neutrophilia as a consequence of more severe cardiac damage in ACS patients compared to SAP patients.[33] However, no heterogeneity between ACS and SAP patients was observed in the relationship between NGAL and IVUS-features of coronary atherosclerosis.

Study limitations

Some limitations of this study merit consideration. This study is currently the largest cohort in which the associations between IVUS plaque characteristics, CysC and NGAL were investigated. Yet, we cannot exclude the possibility of a chance finding with regard to effect modification by kidney function. However, both the cut-off value (based on K/DOQI guidelines) and the study population (no kidney failure/eGFR<30) were chosen a priori. Still, our findings should be considered hypothesis-generating and warrant external validation. Second, kidney function

was determined by the creatinine-based CKD-EPI formula, without direct measurement of GFR. Although the CKD-EPI formula has displayed better performance than the Modification of Diet in Renal Disease (MDRD) equation,[17] it is still possible that a few patients are misclassified. Third, VH-IVUS imaging was limited to a pre-specified target segment of a non-culprit coronary artery. This study design was chosen based on the hypothesis that such a non-stenotic segment reflects coronary wall pathophysiology of the larger coronary tree.[34,35] This hypothesis, on its part, was based on ex-vivo, as well as in-vivo studies using IVUS in patients with myocardial infarction. These studies have demonstrated the presence of TCFAs in places other than the culprit lesion or even culprit artery.[16,36] In fact, we were subsequently able to confirm this hypothesis, by demonstrating that imaging characteristics of the non-culprit artery are associated with increased risk of MACE within the current study population.[34] Therefore, this study design allows us to investigate whether the patient's burden and vulnerability of atherosclerotic disease – as reflected by the phenotype of a non-culprit artery segment – is associated with blood biomarkers.[16] Finally, although the spatial resolution of IVUS-VH is formally too low to detect thin caps, we have demonstrated that VH-IVUS derived TCFA lesions strongly and independently predict the occurrence of MACE within the current study population.[34]

CONCLUSION

This study provides new insights into the role of plasma CysC and NGAL in coronary atherosclerosis. Most importantly, it shows that in patients with normal kidney function, those with higher CysC levels have fewer high-risk coronary lesions, while in patients with impaired kidneys, those with higher CysC have higher risk of MACE. Thus, this study implies that mild-to-moderate kidney dysfunction modifies the relationship between plasma CysC and coronary artery disease. This has not been established before, and it offers an explanation for the difference in findings observed in experimental and epidemiologic studies.With regard to cardiovascular risk prediction, CysC showed predictive capacities when $eGFR_{Cr}$ was below 90 ml/min/1.73m^2, whereas NGAL levels were not predictive of MACE.

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SUPPLEMETARY INFORMATION

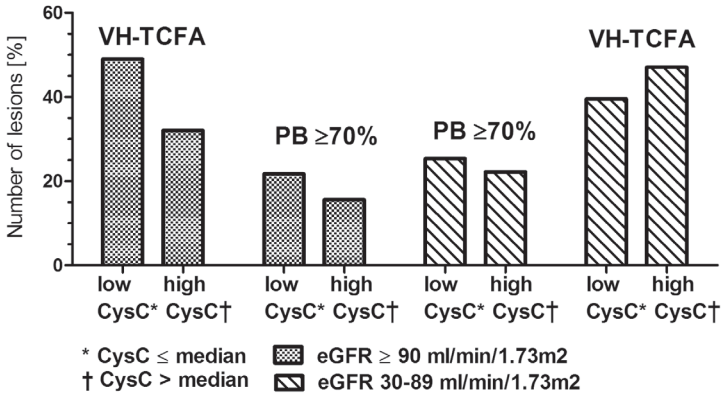


FIGURE S1 Relative number of thin-cap fibroatheroma (VH-TCFA) lesions and lesions with plaque burden (PB)≥70% per strata of kidney function ($eGFR_{cr}$) and plasma cystatin C (CysC) levels above and below median.

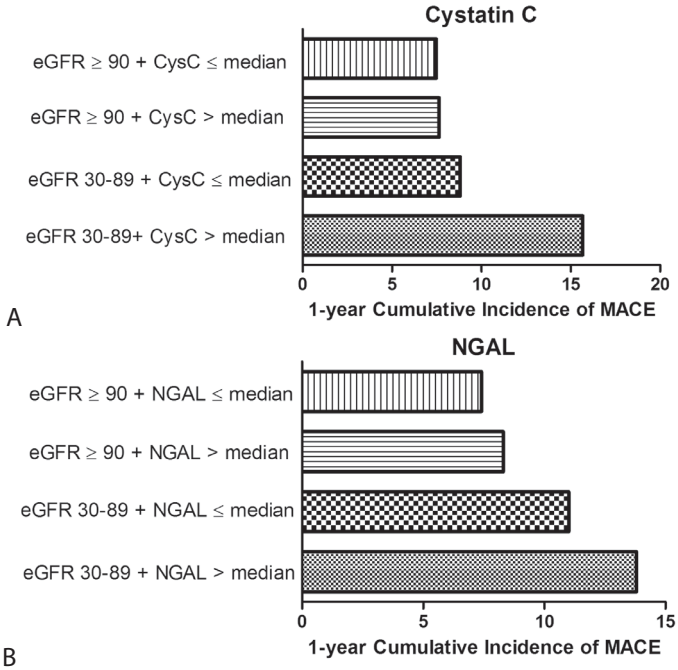


FIGURE S2 1-year cumulative incidence of major adverse coronary events (MACE). A. 1-year MACE per strata of kidney function ($eGFR_{cr}$) and plasma cystatin C (CysC) levels above and below median. **B.** 1-year MACE per strata of kidney function ($eGFR_{cr}$) and plasma NGAL levels above and below median.

TABLE S1 Plasma cystatin C, NGAL and presence of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB)≥70%, and lesions with minimal luminal area (MLA) ≤4 mm².

	Unadjusted		Multivariable model	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Total population				
VH-TCFA				
Cystatin C[a]	0.93 (0.78-1.10)	0.38	0.89 (0.74-1.08)	0.25
NGAL [b]	0.92 (0.76-1.10)	0.36	0.88 (0.72-1.07)	0.19
Plaque Burden ≥70%				
Cystatin C [a]	0.93 (0.76-1.14)	0.50	0.75 (0.59-0.95)	0.018
NGAL [b]	0.95 (0.74-1.21)	0.67	0.92 (0.71-1.19)	0.51
MLA ≤4mm²				
Cystatin C [a]	0.90 (0.75 - 1.08)	0.27	0.79 (0.65-0.98)	0.028
NGAL [b]	1.00 (0.82 - 1.21)	0.95	1.01 (0.82-1.24)	0.90
ACS patients				
VH-TCFA				
Cystatin C [a]	0.86 (0.69-1.07)	0.18	0.80 (0.62-1.03)	0.085
NGAL [b]	0.86 (0.67-1.09)	0.21	0.85 (0.66-1.08)	0.18
Plaque Burden ≥70%				
Cystatin C [a]	0.81 (0.61-1.09)	0.17	0.57 (0.40-0.81)	0.002
NGAL [b]	0.87 (0.62-1.24)	0.45	0.81 (0.56-1.17)	0.26
MLA ≤4mm²				
Cystatin C [a]	1.04 (0.79-1.28)	0.97	0.86 (0.65-1.14)	0.30
NGAL [b]	1.11 (0.85-1.44)	0.44	1.10 (0.84-1.44)	0.50
SAP patients				
VH-TCFA				
Cystatin C [a]	1.05 (0.81-1.37)	0.70	1.06 (0.79-1.42)	0.70
NGAL [b]	0.96 (0.70-1.30)	0.78	0.95 (0.69-1.31)	0.75
Plaque Burden ≥70%				
Cystatin C [a]	1.04 (0.78-1.40)	0.77	1.00 (0.72-1.38)	0.97
NGAL [b]	1.01 (0.77-1.58)	0.60	1.05 (0.73-1.52)	0.79
MLA ≤4mm²				
Cystatin C [a]	0.77 (0.58-1.02)	0.071	0.73 (0.53-0.99)	0.044
NGAL [b]	0.91 (0.67-1.24)	0.55	0.90 (0.65-1.24)	0.51

ACS, acute coronary syndrome; SAP, stable angina pectoris. Multivariable model: adjusted for age, gender, diabetes mellitus, hypertension, indications for angiography, C-reactive protein. [a] Odds ratio (OR) per standard deviation increase ln-transformed cystatin C with 95% confidence interval (CI). [b] Odds ratio (OR) per standard deviation increase in NGAL with 95% confidence interval (CI).

TABLE S2 Plasma cystatin C, NGAL and segment plaque volume, burden and VH-tissue types as determined by VH-IVUS, in the total population and stratified by indication for angiography.

	Cystatin C [a]		NGAL [b]	
	β coefficient (95% CI)	p-value	β coefficient (95% CI)	p-value
Total population				
Plaque burden [c]	0.03 (-0.05 – 0.11)	0.44	-0.02 (-0.11 – 0.07)	0.67
Plaque volume [c]	0.04 (-0.31 – 0.39)	0.83	-0.05 (-0.44 – 0.34)	0.79
FI (%)	-0.46 (-1.42 – 0.50)	0.35	0.52 (-0.54 – 1.58)	0.33
FF (%) [c]	0.01 (-0.07 – 0.09)	0.86	0.05 (-0.04 – 0.13)	0.29
NC (%)	-0.17 (-0.83 – 0.50)	0.62	-0.56 (-1.29– 0.17)	0.13
DC (%) [c]	0.09 (-0.01 – 1.19)	0.072	-0.07 (-0.18 – 0.04)	0.22
ACS patients				
Plaque burden [c]	0.01 (-0.10 – 0.12)	0.89	-0.05 (-0.16 – 0.07)	0.43
Plaque volume [c]	-0.22 (-0.68 – 0.24)	0.35	-0.27 (-0.77 – 0.23)	0.29
FI (%)	-0.20 (-1.50 – 1.10)	0.76	0.54 (-0.87 – 1.95)	0.45
FF (%) [c]	-0.05 (-0.16 – 0.06)	0.39	0.08 (-0.04 – 0.20)	0.18
NC (%)	-0.19 (-1.12– 0.74)	0.69	-0.98 (-1.98 – 0.02)	0.055
DC (%) [c]	0.12 (-0.01 – 0.24)	0.079	-0.06 (-0.20 – 0.08)	0.39
SAP patients				
Plaque burden [c]	0.05 (-0.07 – 0.16)	0.41	0.07 (-0.06 – 0.21)	0.29
Plaque volume [c]	0.35 (-0.20 – 0.90)	0.21	0.39 (-0.25 – 1.02)	0.23
FI (%)	-0.66 (-2.09 – 0.76)	0.36	0.03 (-1.61 – 1.67)	0.97
FF (%) [c]	0.71 (-0.04 – 0.18)	0.22	0.03 (-0.10 – 0.16)	0.64
NC (%)	-0.08 (-1.03 – 0.86)	0.86	-0.09 (-1.18 – 1.00)	0.87
DC (%) [c]	0.04 (-0.12 – 0.19)	0.64	-0.02 (-0.20 – 0.16)	0.85

ACS, acute coronary syndrome; SAP, stable angina pectoris. FI, fibrous, FF; fibro fatty; NC, necrotic core; DC, dense calcium. [a] Unadjusted β per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) [b] Unadjusted β per standard deviation increase in NGAL with 95% confidence interval (CI). [c] Square root transformed.

TABLE S3 Plasma cystatin C, NGAL and composite endpoint of all-cause mortality/acute coronary syndrome(ACS) in total population and stratified by kidney function ($eGFR_{Cr}$).

All-cause mortality/ ACS	Unadjusted		Model 1		Model 2	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
Total population						
Cystatin C [a]	1.67(1.24-2.27)	<0.001	1.51 (1.08-2.10)	0.015	1.24 (0.88-1.77)	0.19
NGAL [b]	1.20 (0.85-1.71)	0.30	1.17 (0.81-1.70)	0.40	1.11 (0.77-1.61)	0.56
$eGFR_{Cr}$ ≥90 ml/min/1.73m²						
Cystatin C [a]	1.39 (0.72 – 2.65)	0.32	1.33 (0.72 – 2.48)	0.36	1.11 (0.54-2.25)	0.78
NGAL [b]	1.04 (0.57-1.89)	0.89	1.12 (0.63-1.98)	0.69	1.08 (0.56-2.11)	0.81
$eGFR_{Cr}$ 30-89 mL/min/1.73 m²						
Cystatin C [a]	1.81 (1.23-2.66)	0.003	1.73 (1.17-2.55)	0.006	1.59 (1.01-2.50)	0.04
NGAL [b]	1.26 (0.80-1.98)	0.31	1.22 (0.77-1.94)	0.39	1.18 (0.74-1.87)	0.48

Model 1: adjusted for the age, gender, indication for angiography; **Model 2**: model 1 + diabetes mellitus, hypertension, C-reactive protein. Multivariable adjustment was constrained by the limited number of clinical endpoints.

[a] Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI).

[b] Hazard ratio (HR) per standard deviation increase in NGAL with 95% confidence interval (CI).

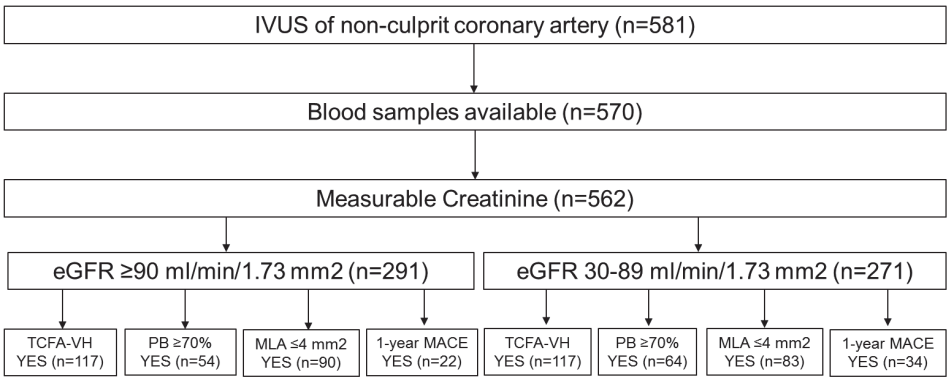


FIGURE S3 Absolute numbers of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB)≥70%, lesions with minimal luminal area (MLA)≤4 $mm^{2,}$ and 1-year major adverse coronary events (MACE) per strata of kidney function ($eGFR_{Cr}$).

TABLE S4 Plasma cystatin C, NGAL and major adverse coronary events (MACE) and the composite of all-cause mortality/acute coronary syndrome (ACS), stratified by indication for angiography.

	Unadjusted		Model 1		Model 2	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
ACS patients						
MACE						
Cystatin C [a]	1.41 (1.01-1.98)	0.047	1.24 (0.85-1.81)	0.27	1.10 (0.73 – 1.65)	0.66
NGAL [b]	1.17 (0.80 – 1.70)	0.43	1.17 (0.80 – 1.72)	0.42	1.14 (0.76 – 1.69)	0.53
All-cause mortality/ACS						
Cystatin C*	1.61 (1.15 – 2.40)	0.007	1.47 (0.98 – 2.20)	0.06	1.23 (0.79 – 1.91)	0.37
NGAL [b]	1.33 (0.89 – 1.98)	0.16	1.35 (0.89 – 2.03)	0.15	1.33 (0.86 – 2.06)	0.20
SAP patients						
MACE						
Cystatin C [a]	1.39 (0.98 – 1.97)	0.07	1.35 (0.92-1.98)	0.12	1.25 (0.85-1.84)	0.26
NGAL [b]	1.18 (0.77 – 1.80)	0.44	1.10 (0.72 – 1.69)	0.66	1.08 (0.70 – 1.64)	0.73
All-cause mortality/ACS						
Cystatin C [a]	1.71 (1.02 – 2.88)	0.042	1.61 (0.90 – 2.87)	0.11	1.40 (0.76 – 2.59)	0.28
NGAL [b]	0.84 (0.42 – 1.70)	0.64	0.79 (0.39 – 1.61)	0.52	0.78 (0.38 – 1.58)	0.49

ACS, acute coronary syndrome; SAP, stable angina pectoris. **Model 1**: adjusted for the age, gender; **Model 2**: model 1 + diabetes mellitus, hypertension, C-reactive protein.
[a] Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI).
[b] Hazard ratio (HR) per standard deviation increase in NGAL with 95% confidence interval (CI).

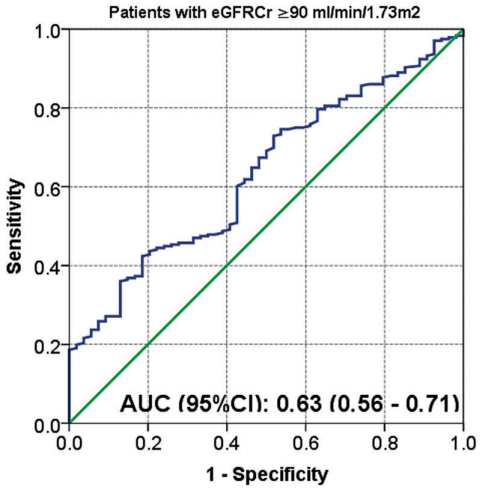


FIGURE S4 Receiver operator characteristic (ROC) curve of plasma cystatin C (CysC) for the prediction of absence of lesion with plaque burden (PB)≥70% in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m². CysC of 773.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have lesion with PB ≥70% (CysC ≥773.0 ng/ml), and those who had (CysC <773.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.

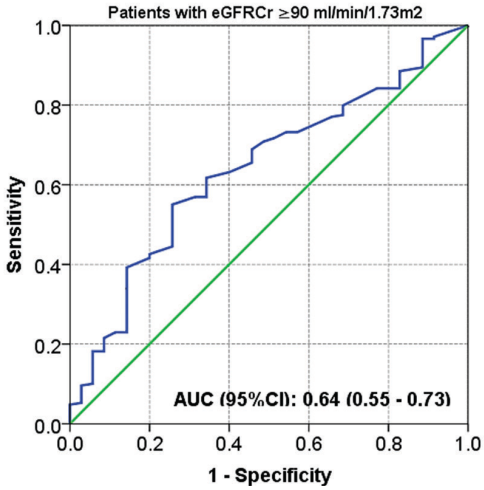


FIGURE S5 Receiver operator characteristic (ROC) curve of plasma NGAL for the prediction of absence of lesion with plaque burden (PB)≥70% in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m². NGAL of 180.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have lesion with PB≥70% (NGAL ≥180.0 ng/ml) and those who had (NGAL <180.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.

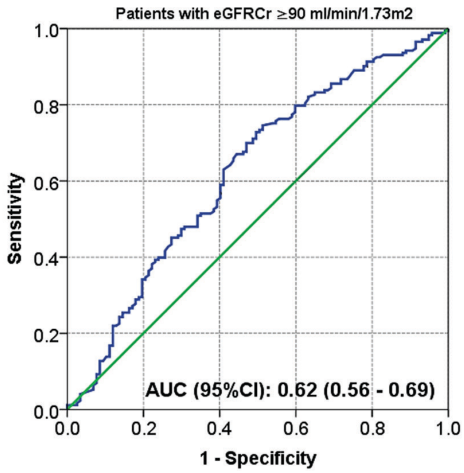


FIGURE S6 Receiver operator characteristic (ROC) curve of plasma cystatin C (CysC) for the prediction of absence of thin-cap fibroatheroma (VH-TCFA) lesion in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m². CysC of 678.5 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have VH-TCFA lesion (CysC ≥678.5 ng/ml), and those who had (CysC <678.5 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.

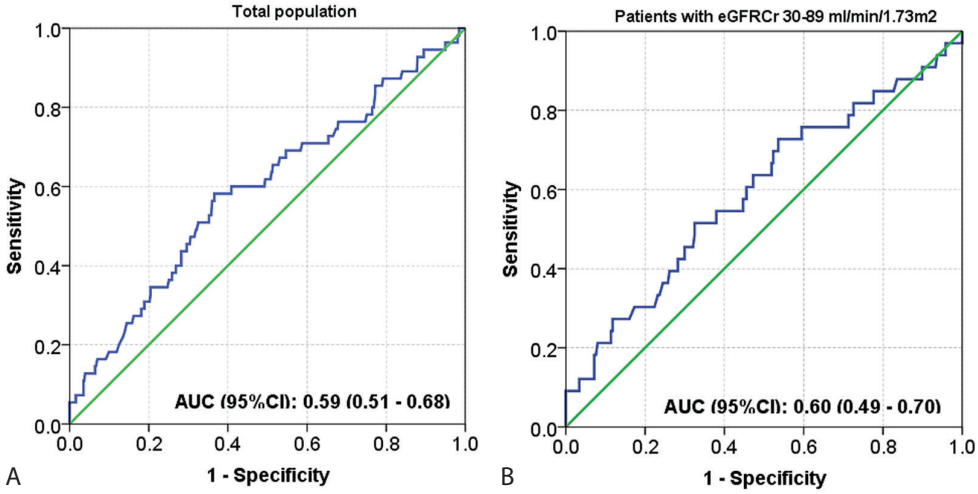
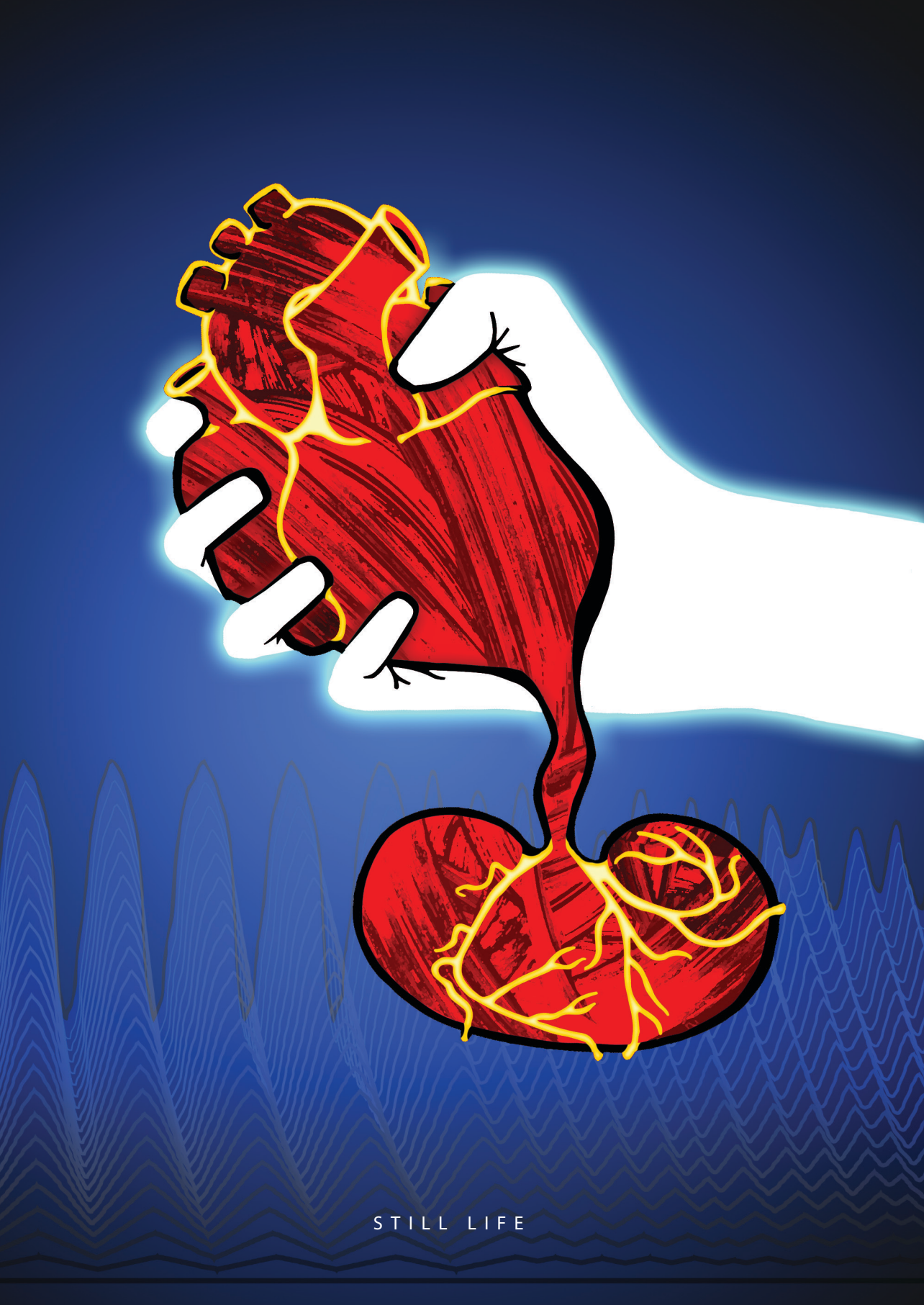


FIGURE S7 Receiver operator characteristic (ROC) curve for plasma cystatin C (CysC) for the prediction of the occurrence of major adverse coronary events (MACE) in total population and in patients with $eGFR_{Cr}$ 30-89 ml/min/1.73m². CysC of 849.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who developed MACE (CysC ≥849.0 ng/ml) and those who did not (CysC <849.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.


STILL LIFE

Part IV

LESSONS LEARNED FROM CLINICAL PRACTICE

CHAPTER 11

Real-life use of Neurohormonal Antagonists and Loop Diuretics in Chronic Heart Failure Analysis of Serial Biomarker Measurements and Clinical Outcome

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ABSTRACT

Background

We determined the temporal effects of neurohormonal antagonists and loop diuretics on serially assessed cardio-renal biomarkers, functional status, and clinical outcomes in patients with chronic heart failure (CHF) with reduced ejection fraction.

Methods

In 250 CHF patients, we measured 3-monthly in blood: NT-proBNP, troponin T, C-reactive protein, creatinine, cystatin C; and in urine: N-acetyl-beta-D-glucosaminidase and kidney-injury-molecule-1.

Results

ACE-inhibitors/ARB were inversely associated with cardiac impairment, inflammation and renal tubular damage, but not with glomerular dysfunction. Diuretics were associated with worse biomarker profiles and with a hazard ratio for adverse clinical outcome of 1.12 (95%CI:1.03–1.22) per 40 mg higher doses. ACE-inhibitors/ARBs were more frequently down-titrated and diuretics more frequently up-titrated in patients who experienced endpoints than in those who did not.

Conclusions

In conclusion, decrease or withholding of ACE-inhibitors/ARBs solely based on glomerular function is not justified because of the beneficial effects on the heart, inflammation, and renal tubules. Higher and increase in diuretic doses mark progression towards end-stage CHF.

INTRODUCTION

In randomized clinical trials (RCTs), neurohormonal antagonists significantly reduce mortality in chronic heart failure (CHF) with reduced ejection fraction.[1-4] In clinical practice, however, optimization of neurohormonal antagonist doses to guideline recommendations is often not reached.[5] Moreover, the temporal effects of dose adjustments of these agents during clinical follow-up of "real-life" patients with CHF are uncertain.

Although guidelines also recommend the use of loop diuretics due to their beneficial effect on symptoms and signs of congestion, no large RCTs have been conducted to prove their efficacy on survival.[6] While longitudinal data on the temporal effects of loop diuretics are absent, studies using cross-sectional data have suggested that the loop diuretics are associated with reduced survival.[7-9] Yet, it is unclear whether this association between poor survival and non-randomized use of diuretics is causal or a reflection of the progressive underlying disease with progressive congestion.[7] Hence, higher doses of loop diuretics will be given to the patients with more severe CHF. However, excessive diuresis may also lead to excessive neurohormonal activation and renal dysfunction, thereby potentially increase mortality.[10,11]

For these reasons identifying the temporal effects of neurohormonal antagonists and loop diuretics on serially assessed patients' functional status and multiple cardio-renal biomarkers, could help to better use of these agents and potentially improve outcomes. The multiple-biomarker strategy enables us to investigate simultaneously the effects of HF medication doses on the evolution of different pathophysiological processes (myocardial stretching and damage, inflammation, renal injury and dysfunction) that occur in CHF regardless of its underlying cause. Similarly, serial measures enable us to control for time-varying health status of patients, thereby providing less biased risk estimates.

In this prospective longitudinal study, our aim was (1) to determine the temporal effects of neurohormonal antagonists and loop diuretics on serially assessed New York Heart Association (NYHA) functional classification, natriuretic peptide NT-proBNP, cardiac troponin T (hs-cTnT), C-reactive protein (CRP), creatinine and cystatin C, and urinary N-acetyl-ß-D glucosaminidase (NAG) and kidney-injury-molecule (KIM)-1, at predefined 3-month intervals during ≥ 2-year outpatient follow-up; (2) to investigate the temporal associations between dose adjustments of these HF medications and clinical outcomes.

MATERIALS AND METHODS

The Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (BioSHiFT) is a prospective observational cohort of patients with CHF, conducted in Erasmus MC, Rotterdam, and Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands. Patients were included if aged ≥18 years, capable of understanding and signing informed consent, and if CHF had been diagnosed ≥3 months ago according to European Society of Cardiology guidelines (Figure S1).[12,13] Patients were ambulatory and stable, i.e., they had not been hospitalized for HF in the past three months. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients. This investigation comprised 263 stable CHF patients enrolled during the first inclusion period (October 2011 until June 2013). Since the effect of certain HF medications, such as RAAS inhibitors, is less firmly established in HFpEF patients than in HFrEF patients, and since 95% of the study population had HFrEF, in this paper we focused on the HFrEF patients (n=250). However, all analyses were also repeated in the full cohort (n=263).

Baseline assessment

All patients were evaluated by research physicians, who collected information on HF-related symptoms, NYHA classification, and performed a physical examination, including blood pressure, heart rate and body mass index. Information on HF etiology, left ventricular ejection fraction, cardiovascular risk factors, medical history and medical treatment was retrieved primarily from hospital records and was checked in case of ambiguities. History of myocardial infarction (MI), percutaneous coronary intervention (PCI), coronary artery bypass grafting (CABG), valvular heart disease, atrial fibrillation or other arrhythmias, cerebrovascular accident (CVA), diabetes mellitus, hypercholesterolemia, hypertension, and COPD were defined as a clinical diagnosis of these conditions, as reported by the treating physician in the medical chart.

Study follow-up and endpoints

Study follow-up visits were predefined and scheduled every 3 months (±1 month was allowed), with a maximum of 10 study follow-up visits (for details see Figure 1 and Table S2). At each study follow-up visit, a research physician performed a

short medical evaluation and collected samples. In parallel, all patients completed their standard outpatient clinic visits at their treating physicians' offices. Treating physicians were unaware of the biomarker results. All medication changes and occurrence of adverse cardiovascular events since the previous visit were recorded in electronic case report forms.

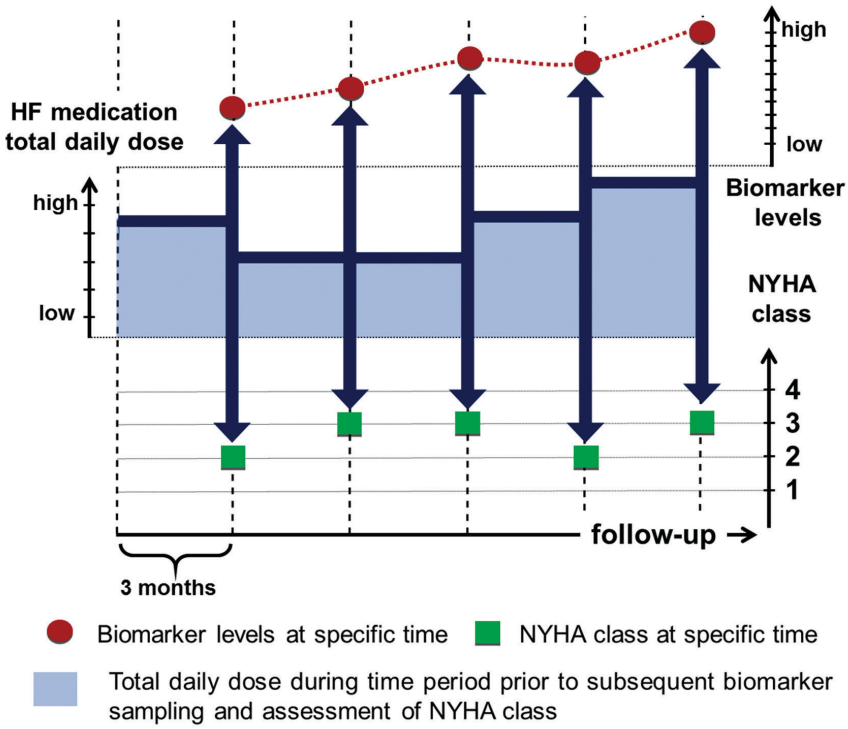


FIGURE 1 Schematic depiction of the analysis of the temporal lagged effects of HF medication doses on NYHA functional classification and biomarker profiles during follow-up. Study follow-up visits were predefined and scheduled every 3 months (X-axis). At these visits a research physician performed a medical evaluation, assessing NYHA functional class (green rectangle), and collecting blood and urine samples for biomarker measurement (red dots). All HF medication changes that had occurred after the previous visit were recorded and calculated as total daily equivalent doses (light blue area); subsequently these doses were related to NYHA class and biomarker profiles at the next outpatient visit (dark blue arrows; temporal lagged effect). All patients were followed until they reached the composite endpoint or until they were censored. To account for differences in the moments in time at which sampling was performed in individual patients and the fact that some patients reached the event and some did not, analyses were adjusted for sampling time and whether or not the patient had an event (for details see statistical analyses).

During follow-up, hospitalizations for HF, MI, PCI, CABG, arrhythmias, CVA, cardiac transplantation, left ventricular assist device (LVAD) implantation and mortality were recorded and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

The primary endpoint comprised the composite of cardiac death, cardiac transplantation, LVAD implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. Secondary endpoints included individual components of the primary endpoint, and also MI, PCI, CABG, CVA, and all-cause mortality. Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalization for acute or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with two of the following: BNP or NT-proBNP >3x ULN, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.[12]

Blood and urine analysis

Blood and urine samples were collected and stored at -80°C. Biomarkers were measured batchwise after follow-up was completed. Laboratory personnel was blinded for clinical data and patients outcomes. Serum NT–proBNP and cardiac troponin T were analyzed by electrochemiluminesence immunoassays (Roche Diagnostics, Elecsys 2010, Indianapolis, Indiana, USA) (LLD: 0.6 pmol/L and 3 ng/L respectively). Serum CRP was measured by immunoturbidimetric assay (Roche Hitachi 912 chemistry analyser, Basel, Switzerland) (LLD: 0.3 mg/L). Creatinine was determined by a colorometric test by the Jaffe reaction (LLD: plasma 0,14 mg/dl, urine: 1.56 mg/ml). Plasma CysC was determined by ELISA (R&D systems, Minneapolis, MN) (LLD: 0.1066 μg/mL). Urinary KIM-1 was determined by ELISA (R&D systems, Minneapolis, MN, USA) (LLD: 0.146 ng/mL), and NAG was determined using a substrate p-nitrophenyl N-acetyl-β-D-glucosaminidase at pH 4.5 (Sigma, St Louis, MO, USA) (LLD: 0.485 U/L). All urinary biomarker were normalized to urinary creatinine concentrations to correct for concentration or dilution of urine. Glomerular filtration rate (GFR) was determined by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation that has been validated in HF patients.[14]

Statistical analyses

Categorical data are summarized by numbers and percentages; continuous data when normally distributed by mean ± standard deviation (SD) and when skewed by median and interquartile range (IQR). Differences between patients with the event and event-free patients were evaluated by the Mann-Whitney U test or Student T test.

The total daily doses (TDD) were converted to equivalents according to ESC guidelines[6] (Table S1). Furosemide equivalent dose above 500 mg (n=7) were excluded from the analysis. To calculate per patient the relative number of up-titrations and down-titrations, the number of times the dose was changed (compared with the previous visit) of a particular patient was divided by the total number of this patient's outpatient visits.

Linear mixed-effects (LME) models were applied to estimate the evolution of HF medication doses over time. Intercept and slope were included in the random-effects design matrix. To achieve normal distributions, biomarkers were ^{2}log-transformed and TDD were $\sqrt{}$-transformed for the analyses.

LME models were also applied to assess the temporal effects of HF medication doses at the current visit on NYHA class and biomarkers at the subsequent outpatient visit (i.e., temporal lagged effect) during follow-up (Figure 1). For this analysis, we used only complete data on all variables (medication, NYHA class, and biomarkers) at corresponding time points during follow-up (per patient: a median of 8 time points). The models were adjusted for sampling time (in the fixed- and random-effects part), and whether or not the patient had an event (in the fixed-effect part). To allow direct comparison of the effects of HF medication on different biomarkers, we used Z-scores (i.e., standard deviation differences from their means). Thus, the effects are depicted as per 1SD increase of HF medication.

Time-dependent Cox survival analysis was applied to investigate the associations between HF medication doses and the study endpoints. Analyses were performed univariably, and then adjusted for potential confounders: age, gender, diabetes, and repeatedly assessed NYHA class, NT-proBNP and eGFR during follow-up. Covariates were chosen based on pathophysiological considerations and were limited in number because we took into account the number of events that occurred during follow-up (and required minimum of 10 outcome events per covariate).

All analyses were performed with R Statistical Software Version 3.[15] All tests were two-tailed and p-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics, Follow-up, and Clinical Outcomes

Table 1 shows baseline clinical and biomarker characteristics of the 250 HFrEF patients. Patients who later experienced the endpoint, at baseline were older, more frequently had diabetes, atrial fibrillation, and history of myocardial infraction, and had lower systolic blood pressure, higher NYHA class, and higher levels of NT-proBNP, cardiac troponin T, CRP, cystatin C, and urinary NAG than patients who remained endpoint-free.

During a median (IQR) follow-up of 2.2 (1.4–2.5) years, we drew a median of 9 blood (IQR: 5–10) and 8 urine (IQR: 5–10) samples per patient, and assessed NYHA functional classification and HF medication 9 (IQR: 5–11) times. Of the HFrEF patients, a total of 66 (26%) patients reached the composite endpoint: 53 patients were re-hospitalized for acute or worsened HF, 8 patients died of cardiovascular causes, 3 underwent heart transplantation, and 2 underwent LVAD placement.

TABLE 1 **Baseline characteristics.**

	Total	Composite endpoint reached		p-value
		Yes	No	
	n = 250	66	184	
Demographics				
Age, years*	66 ± 13	69 ± 13	65 ± 12	0.042
Men, n (%)	184 (74)	52 (79)	132 (72)	0.27
Clinical characteristics				
BMI, kg/m^2 *	27.4 ± 4.7	27.3 ± 4.7	27.5 ± 4.7	0.78
Heart rate, b.p.m.*	67 ± 11	68 ± 13	66 ± 11	0.26
SBP, mmHg*	122 ± 21	116 ± 18	123 ± 21	0.021
DBP, mmHg*	72 ± 11	70 ± 10	73 ± 11	0.052
Features of heart failure				
NYHA class III/IV, n (%)	62 (25)	29 (44)	33 (18)	<0.001
LVEF, % *	30 ± 10	29 ± 9	31 ± 10	0.52
Etiology of heart failure, n (%)				
Ischemic	116 (46)	36 (54)	80 (43)	0.12
Hypertension	31 (13)	8 (12)	23 (12)	0.94
Valvular disease	10 (4)	5 (8)	5 (3)	0.08
Cardiomyopathy	63 (25)	13 (20)	50 (27)	0.23

continued

	Total	Composite endpoint reached		p-value
		Yes	No	
n =	250	66	184	
Unknown or Others	30 (12)	4 (6)	26 (14)	
Medical history, n (%)				
Prior MI	95 (38)	32 (48)	63 (34)	0.041
Prior PCI	81 (32)	26 (39)	55 (30)	0.16
Prior CABG	42 (17)	12 (18)	30 (16)	0.73
Atrial fibrillation	97 (39)	33 (50)	64 (35)	0.030
Diabetes	77 (31)	29 (44)	48 (26)	0.007
Hypercholesterolemia	94 (38)	29 (44)	65 (35)	0.22
Hypertension	113 (45)	34 (51)	79 (43)	0.23
COPD	31 (12)	12 (18)	19 (10)	0.10
NT-proBNP (pmol/L) †	133.1(44.9–274.4)	297.4 (176.4–524.6)	93.9 (29.1–205.0)	<0.001
Hs-TnT (ng/L) †	17.7 (9.3–32.8)	30.1 (19.7–48.6)	13.8 (8.2–27)	<0.001
C-reactive protein mg/L †	2.2 (0.9–4.9)	2.9 (1.4–5.4)	1.8 (0.7–4.3)	0.016
Glomerular function markers †				
Creatinine, mg/dl	1.18 (0.99–1.49)	1.32 (1.02–1.51)	1.17 (0.97–1.48)	0.14
eGFR, mL/min/1.73m^2	58 (42–77)	53 (39–73)	60 (44–78)	0.24
Cystatin C, mg/L	0.73 (0.57–0.97)	0.86 (0.70–1.02)	0.70 (0.52–1.18)	<0.001
KDOQI classification, n (%)				
eGFR ≥90	28 (11)	7 (11)	21 (11)	0.59
eGFR 60-89	92 (37)	20 (30)	72 (39)	
eGFR 30-59	110 (44)	33 (50)	77 (42)	
eGFR <30	20 (8)	6 (9)	14 (8)	
Tubular markers †				
NAG, U/gCr [urine]	5.8 (3.7–9.1)	7.9 (5.9–10.8)	5.1 (3.2–8.0)	<0.001
KIM-1, ng/gCr [urine]	488.6 (246.6–935.2)	589.0 (259.6–1802.7)	462.8 (236.2–900.6)	0.14

BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; NYHA class, New York Heart Association class; LVEF, left ventricular ejection fraction; MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; CVA, cerebrovascular accident; TIA, transitory ischemic attack; COPD, chronic obstructive pulmonary disease; ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; eGFR, estimated glomerular filtration rate. * Normally distributed continuous variables are presented as mean±standard deviation (SD), and non-normally distributed variables as median and interquartile range (IQR). Categorical variables are presented as numbers and percentages.†All biomarkers levels were presented as median and interquartile range (IQR).

Associations of temporal changes in repeatedly assessed HF medication doses with temporal changes in NYHA classification and biomarker profiles during follow-up

Figure 2 shows average temporal lagged effects of repeatedly assessed HF medication doses on subsequent NYHA classification and biomarkers profiles during follow-up.

NYHA functional classification

Higher repeatedly assessed furosemide equivalent doses were associated with higher (i.e., worse) NYHA class values during follow-up. At any time-point during follow-up, one SD increase in equivalent dose of furosemide was related to a 0.10 (95% CI: 0.04–0.15) points higher NYHA class (p<0.001) at the next follow-up visit. Conversely, higher doses of carvedilol and enalapril equivalents were associated with lower (i.e., better) NYHA class values during follow-up: one SD increase in equivalent dose of carvedilol with a 0.06 (0.01–0.12) points lower value (p=0.036), and one SD increase in equivalent dose of enalapril with a 0.07 (0.02–0.13) points lower value (p=0.011).

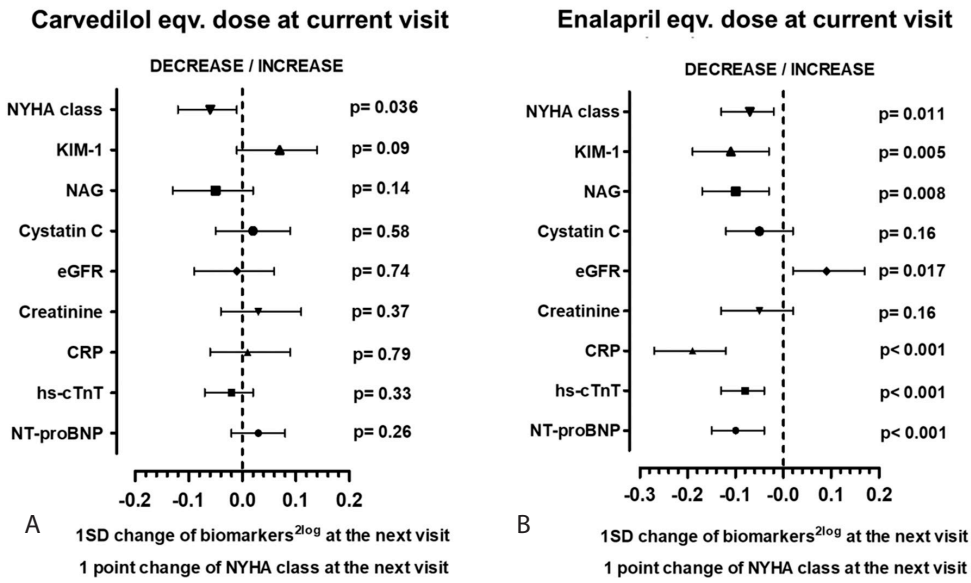
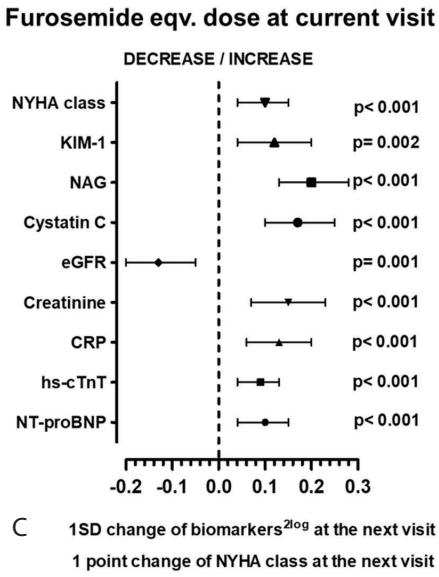
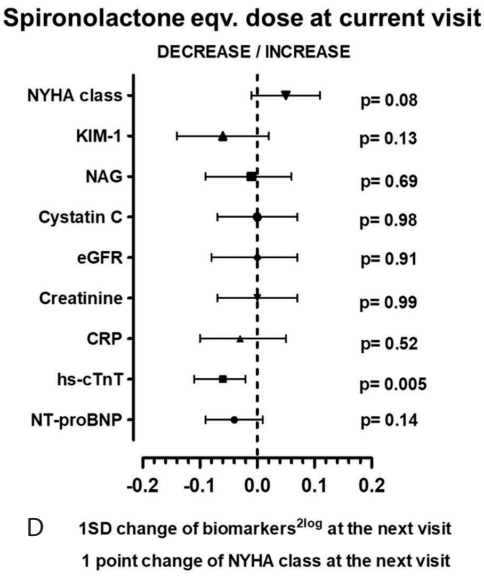


FIGURE 2 Associations of temporal changes in repeatedly assessed HF medication doses with temporal changes in NYHA classification and biomarker profiles in HFrEF patients. The HF medication effects are given as β (95% confidence interval) SD change in ^{2}log-biomarkers levels per 1SD increase in HF medication √-dose. This method of standardization (i.e., per SD) allows





Scale: HFrEF patients	Mean-1SD	Mean	Mean+1SD
HF medication - independent variable			
Carvedilol eqv., mg.	8	32	71
Enalapril eqv., mg.	4	17	39
Furosemide eqv., mg.	9	52	131
Spironolactone eqv., mg.	1	11	30
Biomarkers - dependent variable			
NT-proBNP, pmol/L	24.2	92.4	353.8
hs-cTnT, ng/L	7.4	17.0	39.3
CRP, mg/L	0.7	2.4	7.6
Creatinine, mg/L	0.87	1.21	1.69
eGFR, mL/min/1.73m^2	37	56	84
Cystatin C, µg/mL	0.50	0.74	1.10
NAG, U/gCr [urine]	2.2	4.9	11.1
KIM-1, ng/gCr [urine]	197.2	457.8	1062.7

E

a direct comparison of the effects of HF medication doses on different biomarkers. **A.** Carvedilol equivalent doses, **B.** Enalapril equivalent doses, **C.** Furosemide equivalent doses, **D.** Spironolactone equivalent doses, **E.** Table shows conversion factors for HF medication doses and biomarker levels from logarithmic to linear scale

Myocardial stretching and damage

At any time-point during follow-up, one SD increase in equivalent dose of furosemide was related to a 0.10 SD (0.04–0.15) higher NT-proBNP value ($p<0.001$) at the next follow-up visit, as measured on the ^{2}log scale. As an example on the linear scale, these findings read as follows: in HFrEF patients an increase in furosemide dose from 52 (mean value) to 131 mg (mean+1SD) at the current visit corresponds to an increase in NT-proBNP from 92.4 (mean value) to 118.5 pmol/L (mean value + 0.10 SD) at the next visit. Similarly, one SD increase in equivalent dose of furosemide was also related to a 0.09 SD (0.04–0.13) higher hs-cTnT value ($p<0.001$), as measured on the ^{2}log scale (Figure 2).

At any time-point during follow-up, an increase in equivalent dose of enalapril was associated with lower NT-proBNP and hs-cTnT at the next follow-up visit (one SD higher dose: 0.10 SD [0.04–0.15] lower NT-proBNP values, $p<0.001$; and 0.08 SD [0.04–0.13] lower hs-cTnT values, $p<0.001$) (for details on linear scale see Figure 2).

Inflammation

At any time-point during follow-up, one SD increase in equivalent dose of furosemide dose was related to a 0.13 SD (0.06–0.20) higher CRP value ($p<0.001$) at the next follow-up visit, as measured on the ^{2}log scale. Conversely, higher enalapril equivalent doses were associated with lower CRP levels (one SD higher dose: 0.19 SD [0.12–0.27] lower CRP values, $p<0.001$) (for details on the linear scale see Figure 2).

Renal function and injury

At any time-point during follow-up, one SD increase in equivalent dose of furosemide was related to a 0.13 SD (0.05–0.20) lower eGFR ($p=0.001$), and to 0.17 SD (0.10–0.25) higher cystatin C ($p<0.001$) at the next follow-up visit, as measured on the ^{2}log scale. Associations were also present with greater tubular damage (one SD higher dose: 0.20 SD [0.13–0.28] higher NAG values, $p<0.001$; and 0.12 SD [0.04–0.20] higher KIM-1 values, $p<0.001$) at the next follow-up visit.

At any time-point during follow-up, increase in equivalent dose of enalapril was associated with less tubular damage at the next follow-up visit (one SD higher dose: 0.10 SD [0.03–0.17] lower NAG values, $p=0.008$; and 0.11 SD [0.03–0.19] lower KIM-1 values, $p=0.005$) (for details on the linear scale see Figure 2). Of note, glomerular function improved numerically with higher doses of enalapril equivalents, but this was not statistically significant (Figure 2).

HF medication and clinical outcomes: prevalence of use and frequency of change

At baseline, loop diuretics were given more frequently to patients who experienced adverse events than to event-free patients (97 vs. 89%, p=0.021) (Figure 3). During follow-up, patients who experienced the event had more than twice as many up-titrations of diuretics than event-free patients (8 vs. 3%, p=0.038) (Figure 4). The frequency of unchanged dose during follow-up was numerically, but not statistically, higher in event patients (11 vs. 5%, p=0.10). Importantly, such patients also had more than twice as many down-titrations of ACE-inhibitors/ARBs (5 vs. 2%, p=0.018). In contrast, event-free patients had more up-titrations of ACE-inhibitors/ARBs (0.2 vs. 1.5%, p=0.047).

HF medication and clinical outcomes: average evolutions of total daily doses

At baseline, patients who later experienced adverse events were given significantly higher doses of loop diuretics than patients who remained event-free (94 vs. 43 mg, p<0.001). This difference in average dose remained significant during follow-up (p<0.001), and further increased in the time-period prior to event (Figure 5).

At baseline, the average dose of ACE-inhibitors/ARBs was numerically, but not statistically, lower in patients who experienced the event than in event-free patients (15 vs. 19 mg, p=0.12). However, the average dose significantly decreased in the time-period prior to the event (p=0.015 for the difference during follow-up between patients with events and without events). We also found a tendency towards a simultaneous decrease in ACE-inhibitor/ARB doses and increase in loop diuretic doses in the same patient over time preceding the event. However, this was not the case in event-free patients (Figure S2).

At baseline, patients who experienced adverse events were given, on average, numerically higher doses of mineralocorticoid receptor antagonists (MRAs) than event-free patients (13 vs. 11 mg, p=0.11). However, a decrease in average MRAs dose was observed during follow-up in event patients (Figure 5).

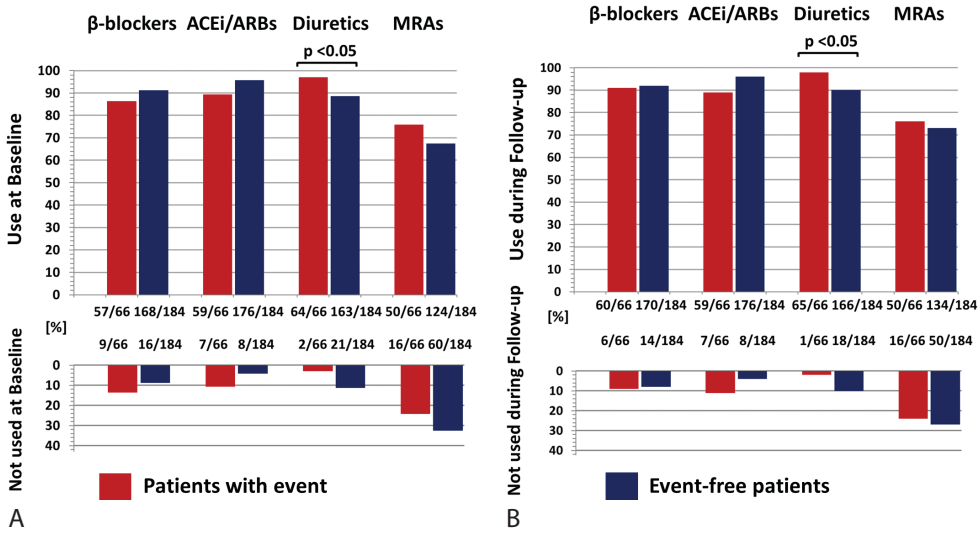


FIGURE 3 Prevalence of HF medication use. Mann-Whitney U test was applied to test the difference between patients who experienced the event and event-free patients. **A.** HF medication use at baseline **B.** HF medication use during follow-up.

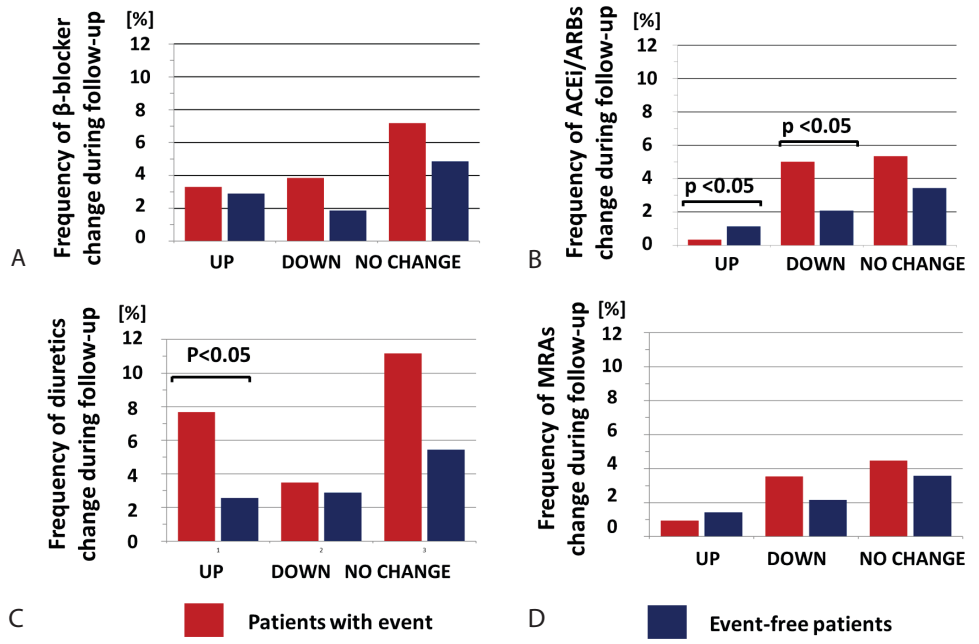


FIGURE 4 Frequency of HF medication change (up-titration/down-titration/no change) during follow-up. Mann-Whitney U test was applied to test the difference between patients who experienced the event and event-free patients. **A.** β-blockers, **B.** ACE-inhibitors/ARBs, **C.** Diuretics, **D.** Mineralocorticoid receptor antagonist (MRAs).

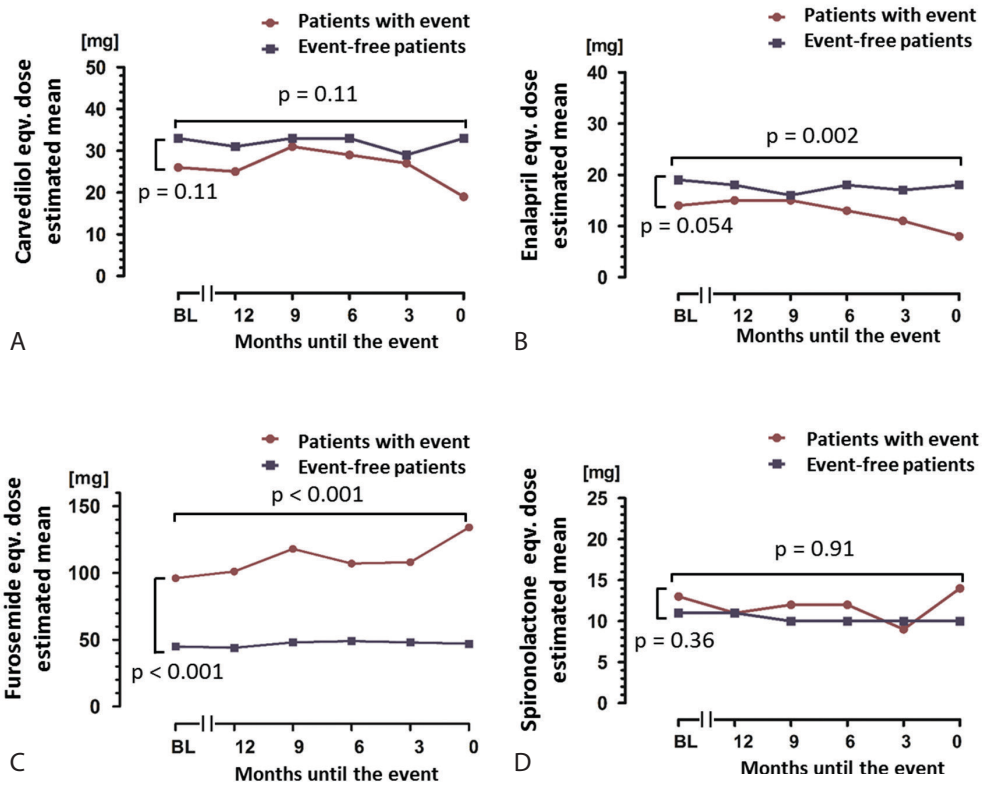


FIGURE 5 Average evolutions of HF medication total daily doses at baseline and during 1-year preceding the event (patients with incident endpoints) or last sample moment (event-free patients). X-axis displays baseline (BL) and the time (months) preceding the event or last sampling moment (time 0). Y-axis displays estimated mean of HF medication total daily dose at each time moment during follow-up. T-test was applied to test the differences at baseline, and mixed-effects models were used to test the difference during follow-up.

HF medication and clinical outcomes: time-dependent survival analysis

Table 2 displays the results of the time-dependent survival analysis. Higher doses of diuretics are independently associated with higher risk of events (per 40 mg increase: HR (95%CI) 1.12 (1.03–1.22), p=0.009). In addition, lower enalapril equivalent doses were univariably associated with increased risk (per 40 mg decrease: 2.41 [1.19–4.88], p=0.014), which did not persist after multivariable adjustment.

TABLE 2 Time-dependent survival analysis of total daily doses in HF medication and the risk of clinical events during follow-up.

HF medication	Unadjusted		Model 1		Model 2	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
β-blockers per 50 mg increase:	0.79 (0.53–1.19)	0.27	0.80 (0.52–1.23)	0.32	0.92 (0.62–1.36)	0.67
ACEi/ARBs per 40 mg decrease:	2.41 (1.19–4.88)	0.014	2.44 (1.20–4.97)	0.014	1.27 (0.65–2.48)	0.48
Loop diuretics per 40 mg increase:	1.24 (1.16–1.33)	<0.001	1.25 (1.17–1.34)	<0.001	1.12 (1.03–1.22)	0.009
MRAs per 25 mg increase:	0.91 (0.58–1.43)	0.68	0.97 (0.61–1.55)	0.90	0.94 (0.59–1.51)	0.81

β-blockers, β-adrenergic receptor blockers; ACE-inhibitors/ARBs, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers; MRAs, mineralocorticoid receptor antagonists. Hazard ratios (HR) with 95% confidence intervals (CI) are given for unadjusted model; **Model 1**: HF medications adjusted for one another; and **Model 2**: HF medications adjusted for age, sex, diabetes + repeatedly assessed: NYHA classification, NT-proBNP and eGFR.

Sensitivity analysis

All above-described analyses were also performed in the full cohort (n=263) which additionally included the HFpEF patients. Results were essentially the same (data not shown).

DISCUSSION

This study is the first to investigate the temporal relationship between medical therapy for HF and detailed biomarker profiles in patients with CHF with reduced ejection fraction. We found that higher ACE-inhibitor/ARB doses are associated with less cardiac impairment, lower inflammation, and less renal tubular damage. No association was observed between higher ACE-inhibitor/ARB doses and glomerular impairment. In contrast, higher loop diuretic doses were associated with worsening of the biomarkers profiles and poor prognosis. We also found that patients who experienced incident clinical events had significantly more down-titrations of ACE-inhibitors/ARBs, and more up-titrations of loop diuretics in the time-period prior to the event. Altogether, these findings challenge the down-titration or withholding of ACE-inhibitors/ARBs solely based on creatinine or eGFR,

and thus carry potential implications for treatment of patients with CHF. Likewise, "renoprotective" treatment targeted at the tubules may be even more effective than treatment aiming at improving renal function in terms of GFR.

CHF and renal dysfunction are highly prevalent, share many risk factors (diabetes, hypertension, hyperlipidemia), and interact to worsen the prognosis.[10,16] Yet, patients with CHF and eGFR <30 ml/min/1.73m^2 have systematically been excluded from RCTs that showed efficacy of ACE-inhibitors/ARBs in reversing cardiac remodeling and improving outcome.[6] Moreover, some reports indicated that use of ACE-inhibitors/ARBs might precipitate acute renal failure.[17,18] This may result in suboptimal dosing of ACE-inhibitors/ARBs in clinical practice as eGFR declines.[19,20] In a recent multicenter study including 11 European countries, lower eGFR remained an independent predictor for suboptimal dosing of ACE-inhibitors/ARBs.[21] In contrast, nephrology guidelines recommend the use of ACE-inhibitors/ARBs in patients with eGFR <30 ml/min/1.73m^2.[22,23] In fact, a pooled analysis of 11 randomized clinical trials has demonstrated a consistent protective effect of ACE-inhibitors on progression of kidney disease.[24] Importantly, ACE-inhibitors impede progression of proteinuria independently of their antihypertensive effect.[25,26] Our study extends these findings by exclusively showing that ACE-inhibitors/ARBs reduce renal tubular damage in patients with CHF. This was demonstrated by two tubular markers (urinary NAG and KIM-1) that were previously found to be strongly associated with tubular damage in patients with acute renal injury,[27] but were also associated with adverse clinical outcomes such as HF re-hospitalisation and mortality in patients with CHF.[28,29] In line with this, our findings indicate that these urinary biomarkers may also be clinically useful for monitoring the kidney's response to ACE-inhibitors/ARBs in patients with CHF. Furthermore, we found, although not significantly, a tendency towards improvement of glomerular function with higher ACE-inhibitor/ARB doses. This is indirectly supported by Frohlich et al., who found that down-titration of ACE-inhibitors/ARBs from higher doses does not improve renal function.[30] Our results also suggest that higher ACE-inhibitors/ARBs doses are associated with lower inflammation in CHF, as shown by repeatedly measured CRP levels. This anti-inflammatory effect of ACE-inhibitors/ARBs,[31,32] although not consistently proven, may be an additional link to improved survival in CHF. This raises the question whether a decrement in renin-angiotensin-aldosterone system (RAAS) blockage is justified solely based on creatinine or eGFR. This issue is especially important in subgroups of patients in whom we found that decrease in ACE-inhibitors/ARBs and increase in diuretics dose occurs in parallel.

As for the effects of diuretics, our time-dependent survival analysis showed that every 40 mg increase in furosemide equivalent dose independently increases the instantaneous risk for 13% (4–19%). This corresponds well with 11% (8–14%) found by Damman et al. in their propensity-matched study of 5011 CHF patients.[7] Yet a 50% reduction in the risk after correction for time-varying health status of patients indicates that substantial confounding by severity of HF is present in the crude risk estimates of loop diuretics.This study is not the first to report an association between these agents and poor prognosis in CHF. However, a unique advantage of this study is frequent repeated assessment both of NYHA functional classification and different cardio-renal biomarkers, which allowed us to thoroughly evaluate the temporal effect of HF medication dosage adjustments in CHF. To this end, we found that higher loop diuretics doses were associated with a deterioration of the complete biomarker profiles, with the largest effect being on the kidneys (glomeruli and tubules). This temporal association between loop diuretics and the levels of glomerular and tubular markers might be of particular importance for optimizing diuretic therapy in such a way that congestion is treated adequately but at the same time, renal injury is not caused. However, in-depth studies on these tubular markers, preferably interventional in nature, are needed to provide definite recommendations on the potential use of biomarker-guided loop diuretic treatment in CHF. Taken together, it is clear that higher, and increase in, loop diuretic doses during follow-up mark progression of CHF. Notably, the effects we found for potassium-sparing diuretics differed from those found for loop diuretics. Higher MRA doses were not significantly associated with adverse biomarker profiles or adverse clinical outcomes. This may in part be attributed to the differences in the mechanisms of action between loop diuretics and MRAs. While the former have been shown to up-regulate the RAAS, the latter result in (beneficial) RAAS blockage. Yet, although efficacy of MRAs has been demonstrated in trials, in our study higher doses only showed a statistically significant association with lower cardiac troponin levels over follow-up; other beneficial effects could not be demonstrated. To this end, in other CHF cohorts, under-prescription of MRAs was found to be a stumbling stone for observing beneficial effects[33-35]

Study limitations

First, although this study was not randomized, its repeated-measures design allows for stronger claims of causality than can be made in previous observational studies. Nevertheless, risk assessment may have been biased by unmeasured confounding although we adjusted for several time-varying variables. Second, our analysis could

not take into account reasons for the dose adjustments. Yet, it is likely that reasons are similar to those identified by Ouwerkerk et al., since our patients were recruited from Dutch hospitals as was the majority of patients in their study.[21] Third, we cannot comment on the anti-proteinuric effect of ACE-inhibitors/ARBs in CHF since we did not measure proteinuria. However, we showed that these agents were associated with less tubular damage which may share similar mechanisms. Importantly, a protective tubular effect was shown by NAG and also by KIM-1, which was qualified as the biomarker for kidney toxicity in preclinical settings (i.e, safety assessment in rats) by the Food and Drug Administration and European Medicines Agency.[36] While we examined a wide array of biomarkers, other biomarkers that were not assessed here may also be relevant and should be investigated in future studies. With the rise of modern –omics technologies, multiple biomarkers that carry potential for heart failure are expected to emerge in the near future. Finally, of note is that the proportion of patients with HFpEF in the current study was low. This may most likely be attributed to the fact that in the Netherlands, most HFpEF patients are treated by the general practitioner or in secondary referral centres, while the current study was performed in two centres which were both tertiary referral centres. We do not deem potential inclusion bias a likely reason for the low proportion HFpEF, because all consecutive patients were screened in both participating centres.

CONCLUSION

In conclusion, decrease or withholding of ACE-inhibitors/ARBs solely based on glomerular function is not justified because of the beneficial effects on the heart, inflammation, and renal tubules. Furthermore, higher and increase in loop diuretic doses during follow-up mark progression towards end stage CHF.

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SUPPLEMENTARY INFORMATION

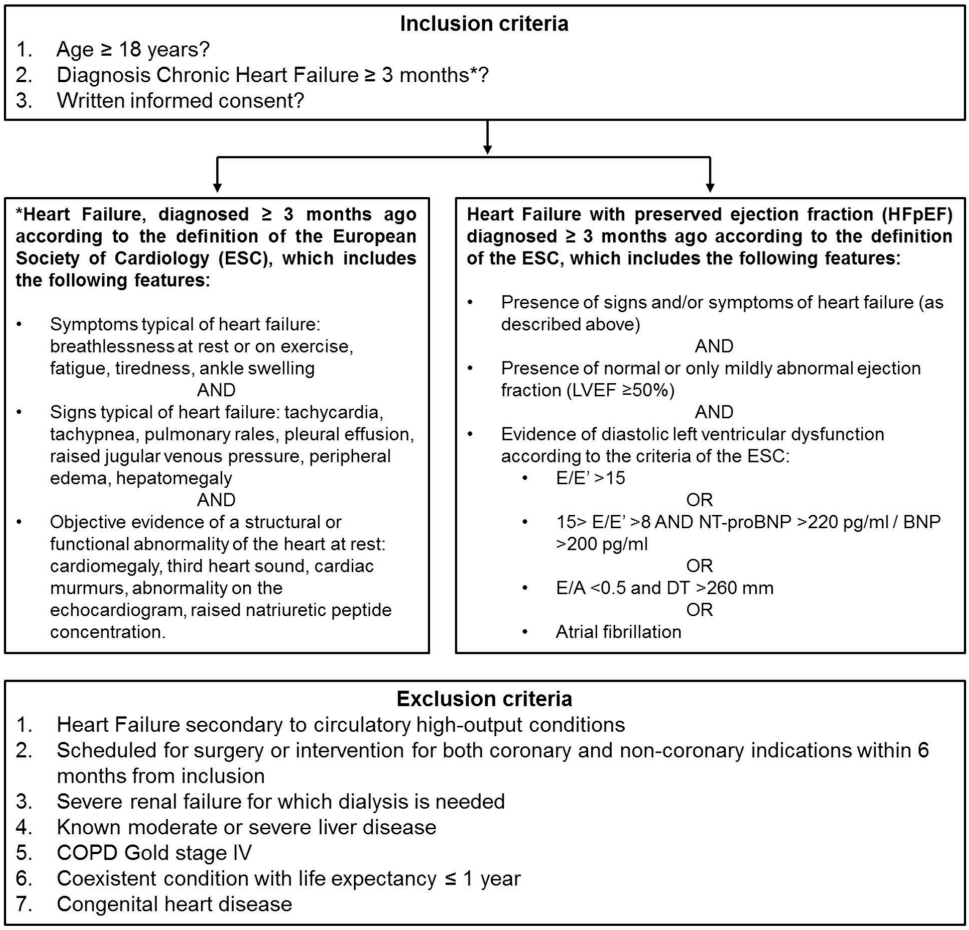

FIGURE S1 Inclusion and exclusion criteria.

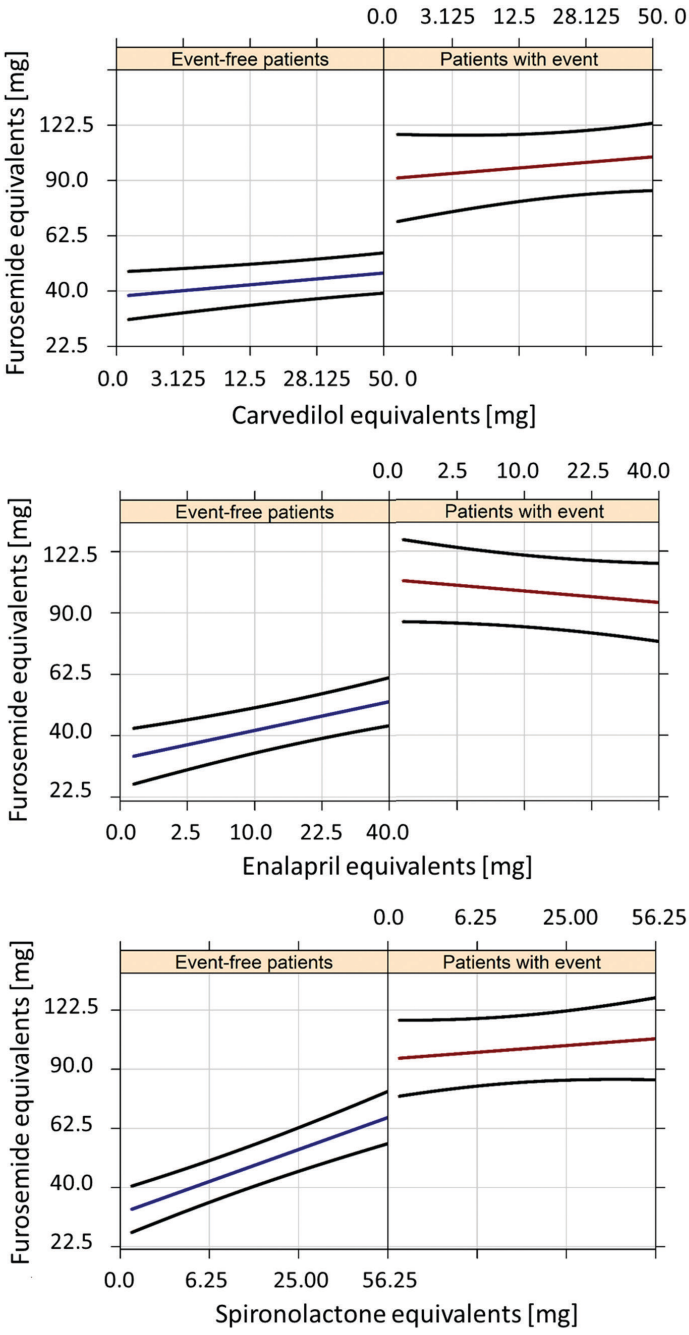

FIGURE S2 Average evolutions of furosemide equivalent doses in relation to equivalent doses of carvedilol, enalapril, and spironolactone within the same patient at the same time during follow-up, stratified by event status.

TABLE S1 **Total daily dose equivalents and conversion factors for ACE-inhibitors/ARBs, β-blockers, MRAs and loop diuretics/thiazides.**

Drug Category	Maximal Dose (Target Dose)	Equivalency Conversion
ACE-inhibitors	**Total Daily Dose (mg)**	**Enalapril Dose Conversion Factor**
Enalapril	40	x 1
Lisinopril	40	x 1
Captopril	150	/ 3.75
Quinapril	40	x 1
Ramipril	10	x 4
Fosinopril	40	x 1
Perindopril	16	x 2.5
Trandolapril	4	x 10
ARB	**Total Daily Dose (mg)**	**Enalapril Dose Conversion Factor**
Candesartan	32	x 1.25
Losartan	50	/ 1.25
Valsartan	320	/ 8
Irbesartan	150	/3.75
β-lockers	**Total Daily Dose (mg)**	**Carvedilol Dose Conversion Factor**
Carvedilol	50	x 1
Bisprolol	10	x 5
Metoprolol tartrate	100	/ 2
Atenolol	50	x 1
Celiprolol	200	/ 4
Labetalol	100	/ 2
Nebivolol	10	x 5
Aldosterone Antagonists	**Total Daily Dose (mg)**	**Spironolactone Dose Conversion Factor**
Spironolactone	25	x 1
Eplerenone	50	/ 2
Loop Diuretic/thiazides	**Total Daily Dose (mg)**	**Furosemide Dose Conversion Factor**
Furosemide	40	x 1
Bumetanide	1	x 40
Torsemide	20	x 2
Hydrochlorothiazide	12.5	x 3.2
Chlorothiazide	36	x 1.44

TABLE S2 Details on HF medication doses and biomarkers data at baseline and during each follow-up visit from the whole study population.

		BL	F-up 1	F-up 2	F-up 3	F-up 4	F-up 5	F-up 6	F-up 7	F-up 8	F-up 9	F-up 10
HF medication*	n[a]	263	251	225	201	209	188	162	140	161	108	88
Carvedilol eqv		7, 32, 73	7, 31, 72	9, 32, 70	8, 33, 74	11, 34, 71	8, 32, 70	8, 31, 70	10, 35, 76	7, 31, 70	8, 30, 67	10, 33, 70
Enalapril eqv		4, 18, 41	4, 17, 41	4, 17, 40	3, 16, 39	4, 17, 40	3, 16, 39	4, 15, 35	3, 15, 36	3, 16, 38	5, 18, 41	3, 14, 34
Furosemide eqv		11, 57, 139	9, 52, 130	10, 54, 134	7, 56, 153	6, 60, 168	6, 56, 157	5, 62, 181	4, 49, 142	4, 55, 165	5, 47, 130	3, 50, 153
Spironolactone eqv		1, 11, 31	1, 11, 31	1, 11, 30	1, 10, 29	1, 11, 30	1, 10, 30	1, 11, 31	1, 10, 28	1, 10, 27	1, 10, 28	1, 9, 27
Serum**	n[b]	263	243	224	199	205	189	168	152	159	104	74
NT-proBNP pmol/L		29.7, 112.8, 428.3	26.8, 102.1, 388.9	25.6, 100.1, 390.7	24.8, 94.3, 358.3	23.7, 90.2, 343.7	24.9, 92.2, 341.5	24.8, 89.1, 320.4	22.7, 85.4, 321.7	24.4, 88.9, 323.8	20.9, 88.9, 378.4	26.4, 88.5, 296.9
Hs-cTnT ng/L		7.8, 18.1, 42.0	7.5, 17.7, 41.6	7.6, 17.9, 41.8	7.2, 17.2, 40.9	7.3, 16.9, 39.3	8.0, 17.4, 37.7	7.5, 17.3, 39.8	6.9,16.0, 37.3	7.5, 16.6, 36.7	7.8, 18.1, 41.8	8.9, 18.1, 37.0
CRP mg/L		0.7, 2.1, 6.6	0.7, 2.3, 7.7	0.7, 2.4, 8.0	0.8, 2.4, 7.3	0.7, 2.4, 7.8	0.8, 2.6, 8.0	0.8, 2.5, 7.9	0.8, 2.3, 6.8	0.9, 2.6, 7.9	0.9, 2.3, 8.8	0.7, 2.6, 9.3
Plasma**	n[b]	263	244	223	199	207	190	167	152	160	105	74
Creatinine mg/dl		0.90, 1.23, 1.68	0.90, 1.22, 1.65	0.89, 1.24, 1.74	0.87, 1.23, 1.74	0.87, 1.23, 1.75	0.88, 1.21, 1.67	0.83, 1.13, 1.53	0.82, 1.12, 1.53	0.87, 1.22, 1.73	0.84, 1.18, 1.65	0.83, 1.25, 1.86
eGFR_mL/ in/1.73m2		37, 55, 83	38, 55, 81	36, 54, 82	36, 55, 83	36, 55, 84	38, 55, 81	42, 61, 88	42, 60, 88	36, 55, 84	38, 57, 85	34, 54, 84
Cystatin C mg/L		0.50, 0.74, 1.09	0.52, 0.79, 1.20	0.55, 0.80, 1.16	0.48, 0.72, 1.09	0.52, 0.74, 1.05	0.51, 0.76, 1.12	0.50, 0.72, 1.02	0.43, 0.68, 1.07	0.50, 0.71, 1.02	0.43, 0.67, 1.03	0.46, 0.70, 1.05
Urine**	n[b]	263	228	206	191	203	183	162	147	155	103	71
NAG U/gCr		2.8, 5.8, 11.9	2.4, 5.4, 12.1	2.3, 4.9, 10.5	2.2, 5.0, 10.9	2.0, 4.9, 11.7	2.1, 4.9, 11.1	1.9, 4.5, 10.6	2.0, 4.7, 10.9	2.1, 4.7, 10.6	1.8, 4.5, 11.1	2.3, 4.6, 9.3
KIM-1 ng/gCr		205.0, 493.8, 1189.1	208.3, 492.3, 1163.8	187.9, 451.3, 1084.2	204.2, 461.3, 1041.8	193.8, 441.3, 1004.9	208.6, 470.7, 1062.0	199.2, 464.1, 1081.2	194.3, 467.4, 1124.6	210.4, 494.4, 1161.8	198.4, 469.0, 1108.8	184.2, 432.4, 1015.1

BL, baseline; F-up, follow-up; eqv. equivalents. [a] number of times HF medication was assessed per follow-up visit [b] number of measured samples per follow-up visit * Geometric mean value ± 1 standard deviation (SD) (i.e., mean - SD, mean, mean + SD) per follow-up visit of √-transformed medication total daily doses presented on the linear (natural) scale. Results are based on the data from the total study population. ** Geometric mean ± 1 SD (mean - SD, mean, mean + SD) per follow-up visit of [2]log-transformed biomarker values presented on the linear (natural) scale. Results are based on the data from the total study population.

CHAPTER 12

Predictive Value of Right Heart Hemodynamics for Acute Kidney Injury After Heart Transplantation

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ABSTRACT

Background

Acute kidney injury (AKI) frequently occurs after heart transplantation (HTx), but its relation to preoperative right heart hemodynamic (RHH) parameters remains unknown. Therefore, we aimed to determine their predictive properties for postoperative AKI severity within 30 days after HTx.

Methods

From 1984 to 2016, all consecutive HTx recipients (n=595) in our tertiary referral center were included and analyzed for the occurrence of postoperative AKI staged by the Kidney Disease Improving Global Outcome Criteria. The effects of preoperative RHH on postoperative AKI were calculated using logistic regression, and predictive accuracy was assessed using integrated discrimination improvement (IDI), net reclassification improvement (NRI), and area under the receiver operating characteristics curves (AUC).

Results

Postoperative AKI occurred in 430 (72%) patients including 278 (47%) stage-1, 66 (11%) stage-2, and 86 (14%) stage-3. Renal replacement therapy (RRT) was administered in 41 (7%) patients. Patients with higher AKI stages had also higher baseline right atrial pressure (RAP) (median: 7, 7, 8, 11 mmHg, p-trend=0.021), RAP-to-pulmonary capillary wedge pressure (PCWP) ratio (0.37, 0.36, 0.40, 0.47, p-trend=0.009), and lower pulmonary artery pulsatility index (PAPi) values (2.83, 3.17, 2.54, 2.31, p-trend=0.012). Higher RAP and lower PAPi values independently predicted AKI severity (adjusted OR per doubling of RAP 1.16[1.02–1.32], p=0.029; of PAPi 0.85[0.75–0.96], p=0.008). Based on IDI, NRI, and delta AUC, inclusion of these parameters improved the models' predictive accuracy.

Conclusions

Preoperative PAPi and RAP strongly predict the development of AKI early after HTx and can be used as early AKI predictors.

INTRODUCTION

Heart transplantation (HTx) remains the gold standard therapy for patients with end-stage heart failure (HF) improving both their survival and quality of life.[1] Recent advances in immunosuppressive therapy and treatment protocols have significantly improved the long-term outcome in HTx recipients despite the propensity for accepting older donors.[2] However, the short-term outcome during the early postoperative phase has remained complex, affecting both morbidity and mortality.[2,3]

Acute kidney injury (AKI) occurs frequently after HTx ranging from 22 to 76% and carries unfavorable prognosis.[4-7] In addition to anesthesia- and surgery-related factors that can precipitate AKI, postoperatively used nephrotoxic drugs (e.g., CNI) and hemodynamic instability may also lead to AKI.[4]

It is known that the preexisting pulmonary hypertension increases the right ventricular afterload that can lead to the right ventricular failure (RVF).[8] Importantly, RVF can critically diminish renal function by increasing renal venous pressure causing congestive AKI.[9,10] Consequently, the right heart hemodynamic (RHH) parameters have been routinely assessed in all HTx candidates.[11] However, it is unclear how these RHH parameters relate to RVF and, more importantly to AKI early after HTx. Finally, the question remains whether and to what extent is the relationship between preoperative RHH parameters and the occurrence of postoperative AKI explained by the occurrence of RVF along that pathway.

Recently, new composite hemodynamic parameters such as the pulmonary artery pulsatility index (PAPi), the right atrial pressure-to-pulmonary capillary wedge pressure ratio (RAP/PCWP), and diastolic pulmonary gradient (DPG) are considered to be the predictors of RVF.[12-15] However, their relationships with postoperative AKI early after HTx remain unknown.

The aim of this study was to determine the predictive properties of the routine and the novel RHH parameters measured at the time of transplantation listing in relation to AKI early after HTx. Preliminary results have been previously reported.[16]

METHODS

Study population

Data of all consecutive HTx in the Erasmus Medical Center, Rotterdam, have been collected prospectively since the first transplantation in June 1984.[2,4] We included all adult (≥18 years) patients transplanted between 1984 and December 2016. Patients

were excluded if age <18 years at the time of transplantation, were on renal replacement therapy (RRT) before transplantation, died within 48 hours or were re-transplanted within 7 days after transplantation (Figure 1). No patients underwent simultaneous heart-kidney transplantation. Patient data were obtained from the hospital database, electronic records and by chart review.

Immunosuppressive protocol

From 1984 to 1999, the immunosuppressive therapy consisted of calcineurin inhibitor (CNI) cyclosporine A and tacrolimus thereafter. In patients who did not receive induction therapy, CNI was initiated peri-operatively or immediately after HTx. The induction therapy was used to delay the starting of CNI, especially in patients with already impaired kidneys and/or postoperative hemodynamic instability, to postpone the CNI nephrotoxicity.[4] The induction therapy consisted of anti-CD3 (1987-1994), anti-IL2 (1987-1994), horse polyclonal anti-thymocyte globulin (1987-2008), and rabbit anti-thymocyte globulin (2008 and thereafter).

Preoperative right heart catheterization parameters

All HTx candidates underwent right heart catheterization during the screening for transplantation listing. If a patient's clinical status deteriorated with suspicion of pulmonary hypertension while on the waiting list, an additional catheterization was performed where the most recent data prior to HTx were used for our analysis. Procedural data were extracted from the catheterization reports and included the following parameters: RAP, PCWP, pulmonary artery (PA) systolic, diastolic and mean pressures, systemic arterial systolic, diastolic and mean pressures, cardiac output, pulmonary vascular resistance, and systemic vascular resistance, PAPi, transpulmonary gradient (TPG), and DPG (Figure 2).[13,15]

Renal function assessment

Serum creatinine was measured as part of routine clinical care at baseline, daily from postoperative day 0 until day 7, and at 1, 3, 6, 9 and 12 months. Baseline creatinine was defined as the most recent outpatient value up to 6 months before transplantation. If unavailable, creatinine values at hospital admission were accepted as the baseline. Estimated glomerular filtration rate (eGFR) was assessed by the CKD-EPI equation[17], and categorization was performed by National Kidney Foundation–Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines.[18]

Follow-up and study endpoints

The primary endpoint was AKI severity as defined by Kidney Disease Improving Global Outcome (KDIGO) criteria during the first month after HTx. AKI stage 1 was defined as serum creatinine increased by ≥0.3 mg/dl (≥26.5 μmol/l) within 48 hours or by 1.5–1.9 times baseline; AKI stage 2 as serum creatinine increased 2.0–2.9 times from baseline; and AKI stage-3 as serum creatinine increased 3.0 times from baseline or by ≥4.0 mg/dl (≥353.6 μmol/l) or starting RRT.[19] The time interval between HTx and the RRT was recorded within the first month. RRT requirement at 1-year was evaluated in all survivors.

The secondary endpoints were postoperative RVF and 1-year survival. RVF was defined as need of postoperative RVAD or as reported in the medical reports by the attending physicians. Post-discharge survival status was obtained from our hospital's electronic patient file and was completed for all patients.

Statistical analysis

For reasons of uniformity all continuous variables are presented as median (interquartile range, IQR), and categorical variables are presented as numbers and percentages. The distributions of continuous variables were tested for normality by the Kolmogorov-Smirnov test, and if skewed were ^{2}log-transformed. For continuous variables, the linear trend across AKI stages was performed by analysis of variance (ANOVA) or the Kruskal-Wallis test, when appropriate; categorical variables were tested by the χ^2-trend test.

Ordinal logistic regression analysis was performed to relate perioperative data to postoperative AKI severity (i.e., deterioration to any level of AKI). Covariates that were univariably associated with AKI severity (exploratory $p<0.10$) were entered into a multivariable model, applying proportional odds ordinal regression with full likelihood ratio method. All analyses were performed in the total cohort, and subsequently in the subgroup of patients with RAP≥6 mmHg (previously determined as the cut-off for the opening of the collapsed vein).[20,21] A multiplicative interaction between dichotomized RAP and PAPi was also explored.

We assessed predictive accuracy for the most severe AKI (stage 3) before and after adding significant hemodynamic parameters ($p<0.05$) into the clinical model using delta between the areas under the two receiver operating characteristic curves (ROC-AUC), integrated discrimination improvement (IDI), and net reclassification improvement (NRI).[22,23] Clinical variables found to be univariably associated with AKI stage 3 (exploratory $p<0.10$) were entered into a multivariable model using stepwise backward likelihood ratio method. Only clinical predictors with $p<0.05$ in the

multivariable model were used to assess the models' predictive accuracy.

Binary logistic regression analysis was applied to assess the association between RRT administration within 30 days after HTx and chronic RRT dependency at first year, and to relate preoperative data to the onset of RVF.

For 1-year survival, we performed the log-rank test and estimated event-time distributions across AKI stages and temporal RRT requirements using the Kaplan–Meier method. Cox regression analysis was performed to assess hazard ratios with 95% confidence intervals for 1-year survival.

All analyses were performed with a complete dataset using SPSS software (SPSS 20.0; IBMCorp., Armonk, NY) and R-statistical software using packages 'pROC', "Hmisc", and "effects".[22,24,25] All tests were two-tailed, and p-values <0.05 were considered statistically significant.

RESULTS

Incidence and temporal trends of postoperative AKI

From 1984 to 2016, 682 patients underwent HTx at the Erasmus MC, of which 595 patients were included in this study (Figure 1). Of 595 patients, 430 (72%) developed AKI, including 278 (47%) stage 1, 66 (11%) stage 2, and 86 (14%) stage 3 AKI. Of those who developed AKI stage 3, 41 (7%) required RRT which lasted for a median of 7 days (IQR: 5–13) and had a 3.3-times (95%CI: 1.6–6.6, p=0.001) higher crude risk of chronic RRT in the first year than those who did not require such treatment. Figure S1 displays the time distribution for the occurrence of AKI with the highest peaks for all three stages on the seventh day.

We found a tendency towards a higher incidence of overall AKI noticeable in recent years. This tendency was accompanied by a trend in a lower baseline eGFR (median eGFR per six 5-year intervals: 69, 67, 67, 56, 69, 56 years, p-trend <0.001), an increasing incidence of diabetes (0, 3, 10, 9, 13, 13%, p-trend <0.001), and older donors (24, 28, 35, 38, 45, 46 years, p-trend <0.001). We also found a tendency towards a higher incidence of AKI stage 3, but only when defined as a requirement for RRT (Figure S2).

Demographic and perioperative data

Table 1 shows baseline characteristics and perioperative data stratified by AKI stages. Patients who had a higher AKI stage also had a higher baseline BMI (median: 22.6, 23.2, 22.9, 24.2 kg/m^2, p-trend<0.001), a lower baseline eGFR (71, 60, 67, 56 ml/min/1.73 m^2, p-trend<0.001), and more frequent diabetes (4, 7, 8,

13%, p-trend=0.015). They also received a heart from older donors (31, 34, 37, 39 years, p-trend<0.001) with more frequently female gender (46, 46, 58, 59%, p-trend=0.019) and postoperatively were more frequently diagnosed with RVF (7, 5, 12, 28%, p-trend<0.001). These patients had a longer hospital stay (20, 24, 24, 37 days, p-trend<0.001) and were less likely to have received induction therapy (90, 80, 71, 67%, p-trend<0.001). A trend was also seen in higher in-hospital mortality with higher AKI stages (2, 5, 14, 9%, p-trend=0.003).

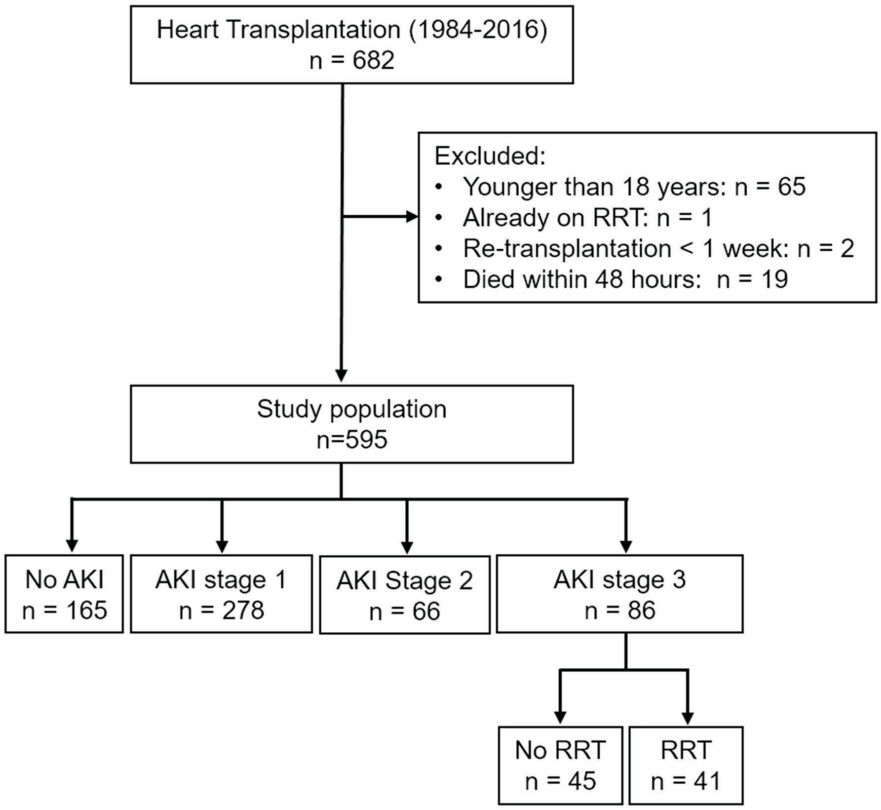

FIGURE 1 Flowchart of study population according to postoperative AKI severity. AKI, acute kidney injury; RRT, renal replacement therapy.

TABLE 1 Baseline characteristics and perioperative data according to postoperative AKI stages.

n (%)	No AKI 165 (28)	AKI Stage 1 278 (48)	AKI Stage 2 66 (11)	AKI Stage 3 86 (14)	p-value
Demographics					
Age, yrs.	51 (45–56)	51 (43–57)	51 (43–57)	48 (41–55)	0.23
Male sex	127 (77)	208 (75)	49 (74)	67 (78)	0.95
BMI, kg/m^2	22.6 (20.1–24.5)	23.2 (21.0–25.2)	22.9 (20.8–25.8)	24.2 (22.1–26.8)	<0.001*
Renal function					
eGFR, ml/min/1.73m^2	71 (58–88)	60 (47–79)	67 (60–79)	56 (43–70)	<0.001*
eGFR ≥90	38 (23)	37 (13)	11 (17)	2 (2)	<0.001*
eGFR 60–89	79 (48)	103 (37)	40 (60)	33 (38)	
eGFR <60	48 (29)	138 (50)	15 (23)	51 (60)	
eGFR 45–59	31 (19)	81 (29)	11 (17)	27(32)	
eGFR <45	17 (10)	57 (21)	4 (6)	24 (28)	
Medical history					
Prior cardiac surgery	45 (27)	89 (32)	15 (23)	25 (29)	0.90
Diabetes mellitus	7 (4)	19 (7)	5 (8)	11 (13)	0.015*
Hypertension	17 (10)	29 (10)	5 (8)	8 (9)	0.65
Donor characteristics					
Age, yrs.	31 (20–42)	34 (22–45)	37 (24–45)	39 (27–49)	<0.001*
Male sex	89 (54)	149 (54)	28 (42)	35 (41)	0.019*
Cause of death					0.61
Trauma	74 (45)	117 (42)	26 (39)	36 (42)	
CVA	83 (50)	149 (54)	38 (58)	4 (51)	
Other	6 (4)	11 (4)	2 (3)	6 (7)	
Unknown	2 (1)	1 (0)	0 (0)	0 (0)	
Time of donor heart ischemia, minutes	165 (139–196)	171 (143–206)	170 (147–195)	176 (150–210)	0.09
Urgency status on waiting list					0.78
Elective	78 (47)	166 (60)	41 (62)	38 (44)	
Urgent	58 (35)	73 (26)	14 (21)	31 (36)	
Unknown	29 (18)	39 (14)	11 (17)	17 (20)	
Preoperative hemodynamic parameters at the time of transplantation listing					
Days before HTx	182 (81–331)	275 (123–545)	273 (117–505)	213 (100–534)	0.15
Heart rate, beats/min	80 (68–100)	80 (70–92)	73 (61–94)	72 (67–90)	0.06
Systolic AP, mmHg	99 (90–106)	97 (90–105)	95 (84–105)	97 (87–107)	0.27
Diastolic AP, mmHg	63 (57–70)	61 (56–69)	62 (54–69)	62 (56–72)	0.91

continued

n (%)	No AKI 165 (28)	AKI Stage 1 278 (48)	AKI Stage 2 66 (11)	AKI Stage 3 86 (14)	p-value
Mean AP, mmHg	74 (68–80)	73 (67–81)	73 (64–81)	75 (66–83)	0.61
CO, L/min	3.8 (3.1–4.6)	4.0 (3.3–4.7)	3.8 (3.2–4.5)	3.8 (3.0–4.5)	0.98
PVR, dynes sec/cm^5	172 (115–230)	149 (96–224)	154 (93–245)	144 (82–226)	0.44
SVR, dynes sec/cm^5	1442 (1192–1764)	1286 (1086–1671)	1398 (1216–1605)	1333 (1042–1630)	0.14
RAP, mmHg	7 (5–12)	7 (4–11)	8 (5–13)	11 (5–17)	0.021*
PA systolic, mmHg	44 (32–55)	42 (30–52)	44 (34–53)	45 (29–59)	0.93
PA diastolic, mmHg	23 (15–30)	21 (14–29)	21 (15–30)	23 (15–29)	0.78
PA mean, mmHg	30 (21–39)	28 (19–36)	27 (21–37)	31 (20–38)	0.84
PCWP, mmHg	21 (14–29)	20 (13–26)	20 (14–27)	22 (13–29)	0.81
TPG, mmHg	8.3 (5.0–11.0)	7.7 (4.3–10.7)	7.2 (4.4–10.3)	7.3 (4.3–10.3)	0.25
DPG, mmHg	1.0 (-2.0–4.0)	1.0 (-2.0–4.0)	0.0 (-2.0–3.0)	0.0 (-2.0–4.0)	0.93
PAPi	2.83 (1.89–5.81)	3.17 (1.61–5.67)	2.54 (1.82–5.60)	2.31 (1.01–4.57)	0.012*
RAP/PCWP ratio	0.37 (0.24–0.57)	0.36 (0.23–0.52)	0.40 (0.25–0.53)	0.47 (0.29–0.74)	0.009*
Preoperative hemodynamic support					
Inotropes	41 (25)	59 (21)	15 (23)	29 (34)	0.16
IABP / ECMO	16 (10)	20 (7)	5 (8)	10 (12)	0.68
LVAD	14 (8)	15 (5)	1 (1)	5 (6)	
Postoperative complications					
Right ventricle failure	11 (7)	14 (5)	8 (12)	24(28)	<0.001*
Re-thoracotomy	12 (7)	18 (6)	9 (14)	12 (14)	0.06
Primary graft failure	3 (2)	4 (1)	4 (6)	2 (2)	0.14
Other [a]	7 (4)	14 (5)	1 (1)	7 (8)	
Immunosuppressive therapy					
Induction therapy	148 (90)	222 (80)	47 (71)	58 (67)	<0.001*
ATG use	89 (54)	151 (54)	29 (44)	46 (53)	0.58
Anti-CD3	45 (27)	41 (15)	5 (7)	5 (6)	<0.001*
Anti-IL2	14 (9)	30 (11)	13 (20)	7 (8)	0.49
Postoperative delay CNI, days[b]	3 (2–5)	3 (1–4)	2 (0–3)	2 (0–5)	0.35
Hospital stay					
Days in ICU	3 (2–4)	3 (2–4)	4 (3–6)	8 (4–14)	<0.001*
Days in hospital	20 (16–29)	24 (17–33)	24 (17–32)	37 (23–58)	<0.001*
In-hospital mortality	4 (2)	15 (5)	9 (14)	8 (9)	0.003*

Due to uniformity, all continuous data are presented as median and inter-quartile range (IQR); all categorical data as number and percentage (%). CO, Cardiac Output; eGFR, estimated glomerular filtration rate; HTx, heart transplantation; AP, arterial pressure; PVR,

pulmonary vascular resistance; SVR, systemic vascular resistance; RAP, right atrial pressure; PA, pulmonary artery; PCWP, pulmonary capillary wedge pressure; TPG, trans-pulmonary gradient; DPG, diastolic pulmonary gradient; PAPi, pulmonary artery pulsatility index; IABP, intra-aortic balloon pump; LVAD, left ventricular assist device; ECMO, extracorporeal membrane oxygenator; ICU, Intensive Care Unit; ATG, anti-thymocyte globulins.
[a] Other includes: perioperative bleeding, cardiac arrest, dosing of inotropes, pacemaker malfunction, acute rejection and instability of unknown cause.
[b] Postoperative delay after heart transplantation before starting calcineurin inhibitor (CNI).
* p-value for linear trend <0.05 is statistically significant.

Relationship of preoperative RHH parameters with postoperative AKI severity

Figure 2 summarizes the investigated hemodynamic parameters. Table 1 shows the values of RHH parameters according to the AKI stages. Patients with a higher AKI stage had also a higher baseline RAP (median: 7, 7, 8, 11 mmHg, p-trend=0.021) and RAP/PCWP ratio (0.37, 0.36, 0.40, 0.47, p-trend=0.009) and lower PAPi values (2.83, 3.17, 2.54, 2.31, p-trend=0.012).

Table 2 and Figure S3 display the associations of the significant RHH parameters with the risk of postoperative AKI severity. In the total cohort, higher RAP and lower PAPi values were associated with AKI severity independently of the patient's BMI, baseline eGFR, diabetes, donor's age and sex, ischemia time of the donor's heart, time from right heart catheterization to HTx, postoperative RVF, and the postoperative use of induction therapy (adjusted OR[95%CI] per doubling: RAP 1.16[1.02–1.32], p=0.029; PAPi 0.85[0.75–0.96], p=0.008). Moreover, we found a significant multiplicative interaction between RAP≥6 mmHg and PAPi values (p-interaction=0.034), indicating even more pronounced association between lower PAPi values and higher probability of AKI severity in patients with elevated RAP (adjusted OR per doubling of PAPi: 0.70[0.56–0.87], p=0.002) (Table 2, Figure S4).

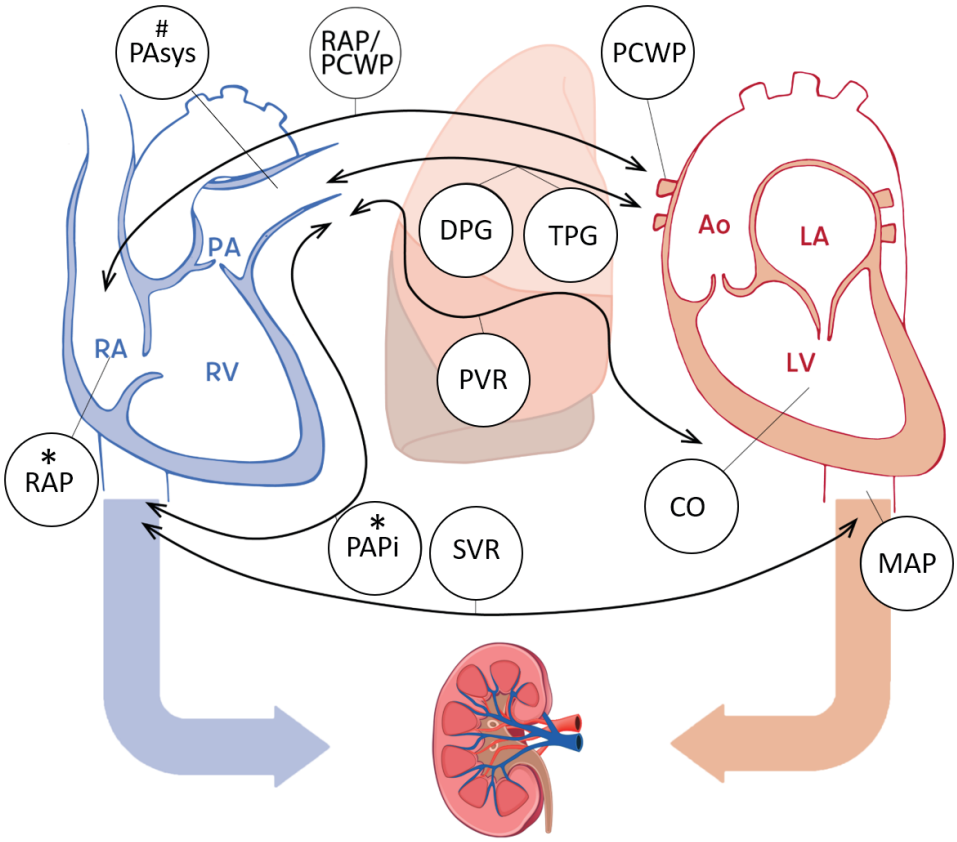

FIGURE 2 Preoperative hemodynamic parameters and their relation to postoperative right ventricular failure and acute kidney injury early (≤30 days) after heart transplantation. An illustration of the assessed hemodynamic parameters of the heart including right atrial pressure (RAP); pulmonary artery (PA) systolic, diastolic, and mean pressures ($PA_{mean\ pressure}$ = [$PA_{systolic}$ + 2*$PA_{diastolic}$] / 3); pulmonary artery pulsatility index (PAPi) (PAPi = [$PA_{systolic\ pressure}$ – $PA_{diastolic\ pressure}$] / RAP; pulmonary capillary wedge pressure (PCWP); RAP/PCWP ratio; transpulmonary gradient (TPG) (TPG = $PA_{mean\ pressure}$ – PCWP); diastolic pulmonary gradient (DPG) (DPG = $PA_{diastolic\ pressure}$ – PCWP); cardiac output (CO); pulmonary vascular resistance (PVR) (PVR = 80 * [$PA_{mean\ pressure}$ – PCWP] / CO); systolic, diastolic, and mean arterial pressure (MAP) (MAP = [$AP_{systolic}$ + 2 * $AP_{diastolic}$] / 3); systemic vascular resistance (SVR) (SVR = 80 * [MAP – RAP] / CO). * indicates significant predictor of acute kidney injury (AKI); # indicates significant predictor of right ventricular failure (RVF); RA indicates right atrium; RV indicates right ventricle; PA indicates pulmonary artery; LA indicates left atrium; LV indicates left ventricle; Ao indicates aorta.

TABLE 2 Associations between preoperative hemodynamic parameters and postoperative AKI severity early after heart transplantation.

	Univariable model		Multivariable model [f]	
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**
Total cohort [a]				
PAPi [c]	0.88 (0.79–0.99)	0.043*	0.85 (0.75–0.96)	0.008*
RAP [c]	1.12 (0.99–1.28)	0.07	1.16 (1.02–1.32)	0.029*
RAP/PCWP [c]	1.19 (1.01–1.39)	0.033*	1.15 (0.98–1.35)	0.10
Heart rate [d]	0.90 (0.81–1.00)	0.06	0.98 (0.87–1.11)	0.75
SVR [e]	0.96 (0.91–1.00)	0.06	0.96 (0.92–1.01)	0.13
Subgroup RAP ≥6 mmHg [b]				
PAPi [c]	0.74 (0.60–0.92)	0.006*	0.70 (0.56–0.87)	0.002*
RAP [c]	1.62 (1.17–2.25)	0.004*	1.78 (1.27–2.50)	0.001*
RAP/PCWP [c]	1.44 (1.06–1.96)	0.019*	1.31 (0.95–1.08)	0.10
Heart rate [d]	0.88 (0.77–1.01)	0.08	0.95 (0.82–1.11)	0.52
SVR [e]	0.98 (0.92–1.03)	0.42	0.99 (0.93–1.05)	0.78

OR (95% CI) indicates proportional odds ratio with 95% confidence interval; for other abbreviations please see table 1. *p-value <0.05 is statistically significant.
[a] total n=595, no AKI = 165, AKI stage 1 = 278, AKI stage 2 = 66, AKI stage 3 = 86.
[b] total n=340, no AKI = 94, AKI stage 1 = 147, AKI stage 2 = 42, AKI stage 3 = 57.
[c] OR are given per doubling of a preoperative hemodynamic parameter. OR are interpreted as the odds of having a more severe renal injury for any level of AKI (stage 3, stage 2, stage 1, and no AKI). For example, if RAP increases from 7 to 14 mmHg (i.e., doubled) the odds of having AKI stage 3 versus combined AKI stages ≤2 and no AKI are 1.12 times greater. Odds of having AKI stages ≥2 versus combined AKI stage 1 and no AKI are 1.12 times greater. Finally, the odds of having AKI of any stage versus no AKI are 1.12 times greater.
[d] OR per 10 units increase in preoperative heart rate (interpretation is the same as under c).
[e] OR per 100 dynes sec/cm^5 increase in preoperative SVR (interpretation is the same as under c).
[f] preoperative hemodynamic parameters were adjusted for all variables with p<0.10 in univariable analysis and included patient's BMI, baseline eGFR, diabetes, donor's age and sex, ischemia time of donor's heart, time from catheterization to HTx, postoperative RVF, and the postoperative use of induction therapy. Associations between these variables and postoperative AKI stages are presented in Table S1.

For predicting AKI stage 3, adding each of the hemodynamic parameters, PAPi and RAP, significantly improved the models' predictive accuracy compared to the best clinical model (PAPi: IDI=0.03, p=0.013 and total continuous NRI=0.320, p=0.007 with 25% reclassification for events and 7% reclassification for non-events; RAP: IDI=0.02, p=0.040 and NRI=0.278, p=0.018 with 25% reclassification for events and 3% reclassification for non-events). In Figure 3, ROC-AUC analysis also showed significant discriminatory improvement (clinical model: AUC 76.1% [95%CI:70.4–81.3], clinical model + PAPi: 79.0% [73.8–83.8], p-delta=0.044; clini-

cal model + RAP: 78.8% [73.6–83.5], p-delta=0.049; PAPi alone: 60.7% [53.8–67.1]; RAP alone: 62.2% [54.6–69.0]). Based on Youden's index, the best cut-off point for predicting AKI stage 3 was PAPi <1.05 and RAP >11 mmHg. Finally, the associations of clinical data with postoperative AKI severity are shown in Table S1.

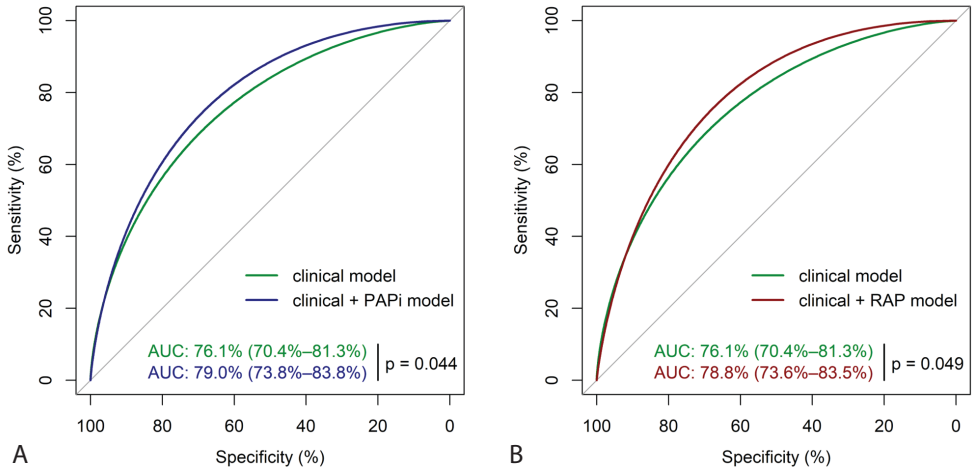

FIGURE 3 The ROC-AUC analysis for the prediction of AKI stage 3. AUC indicates area under the ROC curve with p-value for the difference between different models. Only predictors that remained significant (p<0.05) in the final model were used to assess discriminative power, and those were patient body mass index, baseline eGFR, postoperative right ventricular failure, and the postoperative use of induction therapy. **A.** The ROC curves of clinical model (green) and clinical model + PAPi (blue) for the prediction of AKI stage 3 with AUCs and corresponding 95% confidence intervals; **B.** The ROC curves of clinical model (green) and clinical model + RAP (red) for the prediction of AKI stage 3.

Predictors of RVF and its relation to AKI

Of 595 patients, 57 (10%) experienced RVF early after HTx. Table S2 shows the predictors of early RVF among which the most significant clinical predictors were the patients' impaired baseline eGFR and older donors; higher pulmonary artery systolic pressure was the only preoperative RHH parameter that predicted RVF. Furthermore, the occurrence of RVF was strongly associated with AKI severity (Table S1).

Relationship of AKI with 1-year mortality

In total, 51 deaths occurred during the first year after HTx with a cumulative mortality of 5%, 7%, 15%, and 14% for those without AKI and with AKI stages 1, 2, and 3, respectively (log-rank test, p-trend=0.021; Figure S5A). The cumulative mortality was also higher in patients who received RRT during the first month than in those who had not (22 *vs.* 8%; log-rank test, p=0.001, Figure S5B).

DISCUSSION

To the best of our knowledge, this study is the first to assess the predictive properties of different preoperative hemodynamic parameters in relation to the occurrence of postoperative AKI severity within 30 days after HTx. We found that lower PAPi and higher RAP values predict AKI severity independently of the recipient BMI, baseline eGFR, diabetes, donor age and sex, ischemia time of the donor's heart, time from right heart catheterization to HTx, postoperative RVF, and the use of induction therapy. These hemodynamic parameters routinely collected at the time of transplantation listing could be used to predict AKI severity early after HTx.

Although renal injury after HTx has traditionally been attributed to the impaired arterial perfusion and CNI nephrotoxicity[4], our results illustrate strong evidence of the independent relationship between preoperative right-sided hemodynamics and AKI severity after HTx. One of the potential explanations for this relationship may be the longstanding venous congestion that chronically compromises the kidneys. Subsequently, the kidneys may become more vulnerable to the development of AKI, especially in cases of postoperative hemodynamic instability with hypotensive episodes, or in the settings of de-novo RVF during the adaptation period of the new heart. However, we found that a de-novo RVF significantly contributed to the development of AKI but could not entirely explain the relationships of preoperative RAP and PAPi with the postoperative renal injury. Several pathophysiological mechanisms may be responsible for this peculiar renal vulnerability, including attenuated vascular reflexes, elevated renal interstitial and intra-tubular pressure, activation of the renin-angiotensin-aldosterone system, and chronic venous pressure-induced tubule-glomerular feedback dysfunction.[26,27] Moreover, we found a significant interaction between RAP and PAPi, indicating that probabilities of AKI (especially stages 2 and 3) are markedly increasing with lower PAPi values, but mostly in patients with elevated RAP (≥6 mmHg). These findings are supported by Damman et al., who found that eGFR starts to significantly decline when RAP increases above 6 mmHg.[28] Altogether, it appears that preoperatively compromised right-sided venous pressures deserve clinical attention in the context of postoperative AKI and may be even more important for kidney functioning after HTx than low systemic pressures.

Acute or chronic cardiac dysfunction has negative effects on kidney function and vice versa. This complex cross-talk was recently described as cardio-renal syndrome (CRS).[29] Chronic HF leading to chronic renal congestion in the pre-HTx period is classified as CRS Type-2. On the other hand, early AKI post-HTx could be considered as CRS Type-1. Recognition of post-HTx CRS may provide possibilities of prevention and treatment strategies in the settings of HTx. Importantly, early prediction of postoperative AKI based on preoperative RAP and PAPi could help to timely and

more proactively intervene in the patients who are at high risk, in terms of giving more attention to the perioperative volume overload, postponing the introduction of nephrotoxic CNI, prolonging the support of the right ventricle with inotropes, NO ventilation, and early introduction of pulmonary vasodilators (e.g., sildenafil).[11,27,30-33] Furthermore, these patients could possibly benefit from functional kidney stress tests to assess the renal functional reserve and identify patients who will progress to AKI post-HTx.[34,35] In addition to decreasing values, PAPi can also go in the opposite direction. Hence, the recovery of PAPi values may indicate improvement of right-sided pressure, which may be used to optimize perioperative hemodynamic support to preserve the kidneys from injury. However, prospective studies, preferably interventional in nature, are needed to elucidate this promising concept.

The overall AKI incidence was 72%, which fits at the high-end of the reported incidence range of 22 to 76% in HTx recipients.[4-7] This widespread distribution probably results from the large heterogeneity between studies and deserves closer attention. Previously, most studies reported the older Risk Injury Failure Loss End-stage Renal Disease (RIFLE) criteria or the Acute Kidney Injury Network (AKIN) criteria.[6,36] However, we used the newer KDIGO criteria to define AKI stages.[37] Second, time-intervals for the occurrence of AKI were different from the present study.[4-7,38,39] We targeted one month as the clinically relevant time-interval, whereas previous studies mainly focused on the first week.[4,6,7,38] Our results show that, although all AKI stages peaked on day 7, the risk of AKI remains through the first month. This late postoperative peak of AKI could be attributed to the delay in starting of CNI. For this purpose, we use induction therapy especially in patients with impaired kidneys and/or hemodynamic instability early after HTx to postpone CNI nephrotoxicity. In our data, the use of induction therapy was protective for the occurrence of AKI. Another important and more worrisome observation is that AKI incidence increased in recent years. This is probably explained by an increasing proportion of HTx recipients being listed with more comorbidities such as chronic renal failure and diabetes, older heart donors, and probable changes in clinical management with more pro-active treatments protocols.[2,40]

Study limitations

First, this was a single-center study, and therefore, the clinical management and treatment modalities may differ from other transplant centers. However, patient selection for transplantation listing and cardiac procedures were performed according to generally accepted international criteria.[11] Second, its retrospective nature precluded the inclusion and evaluation of additional parameters such as echocardiography. Furthermore, we were not able to analyze the postoperative RHH parameters due to un-

availability and incompleteness of the historical data. Therefore, postoperative RVF could only been retrieved as reported or the need for postoperative RVAD. Further studies are needed to analyze the incremental value of post-HTx RHH parameters. Third, we did not include urine output in the definition of AKI because these patients were on intensive diuretic therapy, which would lead to misinterpretation of the urine output values. Altogether, this study is currently the largest HTx cohort in which the associations between preoperative hemodynamics and postoperative AKI were investigated.

CONCLUSIONS

Preoperative PAPi and RAP strongly predict the postoperative AKI early after heart transplantation and can be used as early AKI predictors.

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SUPPLEMETARY INFORMATION

TABLE S1 Associations of clinical data with postoperative AKI severity early after heart transplantation.

	Univariable model [a]		Multivariable model [b]	
Clinical data	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
Preoperative				
BMI per 1 kg/m^2 increase	1.10 (1.05–1.15)	<0.001*	1.06 (1.01–1.12)	0.019*
Diabetes	2.00 (1.12–3.57)	0.019*	1.38 (0.73–2.60)	0.32
eGFR per 10ml/min/1.73m^2 decrease	1.18 (1.10–1.26)	<0.001*	1.11 (1.02–1.19)	0.011*
Donor's age per 5 years increase	1.11 (1.05–1.17)	<0.001*	1.06 (1.00–1.13)	0.07
Donor's female gender	1.40 (1.03–1.88)	0.029*	1.19 (0.86-1.66)	0.29
Ischemia time per 30 minutes longer	1.10 (0.99–1.21)	0.07	1.07 (0.96–1.19)	0.25
Time from RHC to HTx per 100 days longer	1.05 (1.01–1.10)	0.022*	1.06 (1.01–1.11)	0.027*
Postoperative				
RVF	3.95 (2.37–6.57)	<0.001*	3.82 (2.22–6.57)	<0.001*
Induction therapy	0.42 (0.29–0.61)	<0.001*	0.29 (0.19–0.44)	<0.001*

BMI indicates body mass index; eGFR indicates estimated glomerular filtration rate; RVF indicates right ventricular failure; RHC indicates right heart catheterization; HTx indicates heart transplantation. OR (95% CI) indicates proportional odds ratio with 95% confidence interval, and are interpreted as the odds of having a more severe renal injury for any level of AKI. [a] Covariates that were found to be univariably associated with AKI severity (p<0.10) were entered into a multivariable ordinal regression model applying the full likelihood ratio method. [b] adjusted for each other. * p-value <0.05 is statistically significant.

TABLE S2 Associations of preoperative clinical and hemodynamic data with postoperative RVF early after heart transplantation.

	Univariable model [a]		Multivariable model [b,c]	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Preoperative clinical data				
BMI per 1 kg/m^2 increase	1.07 (0.99–1.16)	0.10		
Diabetes	2.42 (1.06–5.52)	0.036*		
eGFR per 10ml/min/1.73m^2 decrease	1.22 (1.06–1.41)	0.005*	1.19 (1.02–1.36)	0.031*
Donor's age per 5 years increase	1.21 (1.10–1.34)	<0.001*	1.21 (1.08–1.35)	0.001*
Donor's female gender	2.40 (1.34–4.31)	0.003*		
Preoperative hemodynamic data				
PA systolic per 5 mmHg increase	1.09 (0.99–1.17)	0.07	1.14 (1.03–1.25)	0.008*
TPG per 5 mmHg increase	1.28 (1.00–1.63)	0.05		
IABP or ECMO use	2.21 (1.02–4.82)	0.045*		

OR indicates odds ratio for having a postoperative RVF; 95% CI indicates 95% confidence interval for the corresponding OR; RVF indicates right ventricular failure; BMI indicates body mass index; eGFR indicates estimated glomerular filtration rate; PA systolic indicates pulmonary artery systolic pressure; TPG indicates transpulmonary gradient. [a]All preoperative data from Table 1 were related to the early RVF; only variables with p<0.10 in univariate analysis were reported in this table. [b]Variables that were found to be univariably associated with early RVF (p<0.10) were entered into a multivariable binary logistic regression model applying the stepwise backward likelihood ratio method with a value of p=0.05 as a removal criterion for the final model. [c]AUC for the final multivariable model was 67.8% (95% CI: 59.8–74.7), p<0.001. *p-value <0.05 is statistically significant.

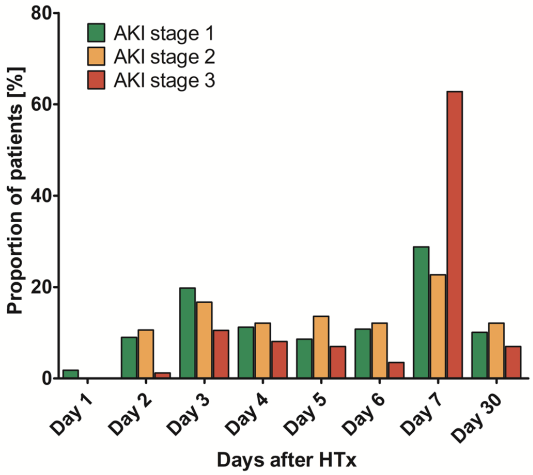


FIGURE S1 Time distribution of AKI occurance according to the severity stage during the first postopearative month. The X-axis depicts time in days; the Y-axis depicts proportion of patients per AKI stage. AKI, acute kidney injury; HTx, heart transplantation.

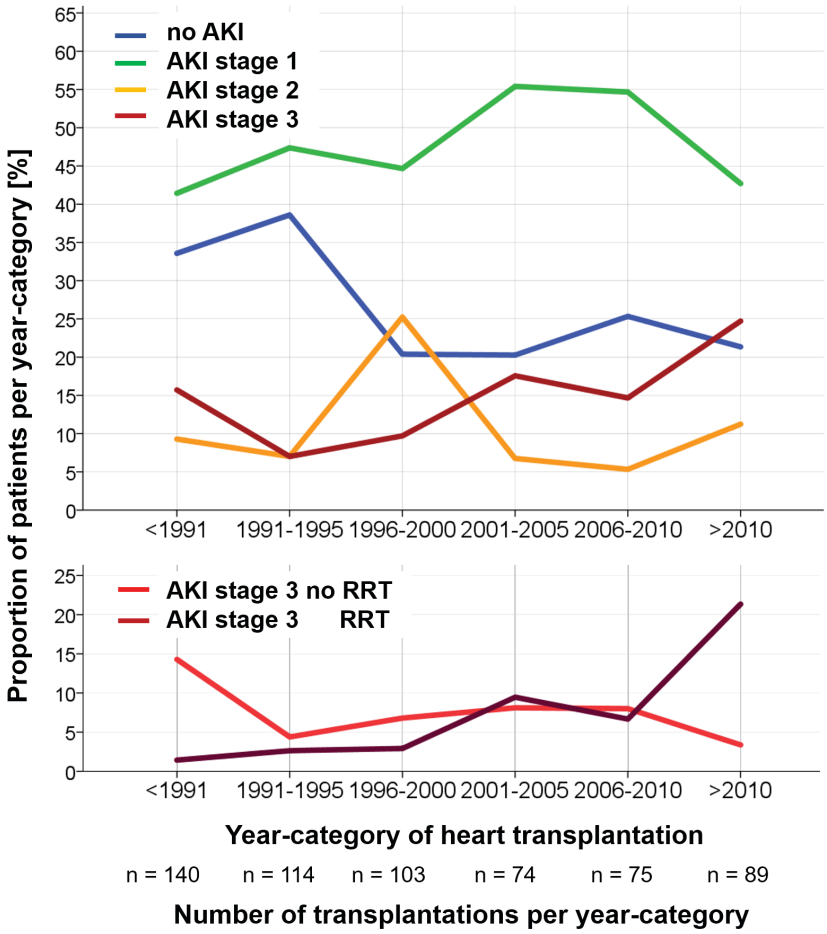


FIGURE S2 Temporal trend in the incidence of postoperative AKI according to severity stage during the period from 1984 until 2016. The X-axis depicts 5-year intervals. The Y-axis depicts proportion of patients per 5-year category according to AKI stage.

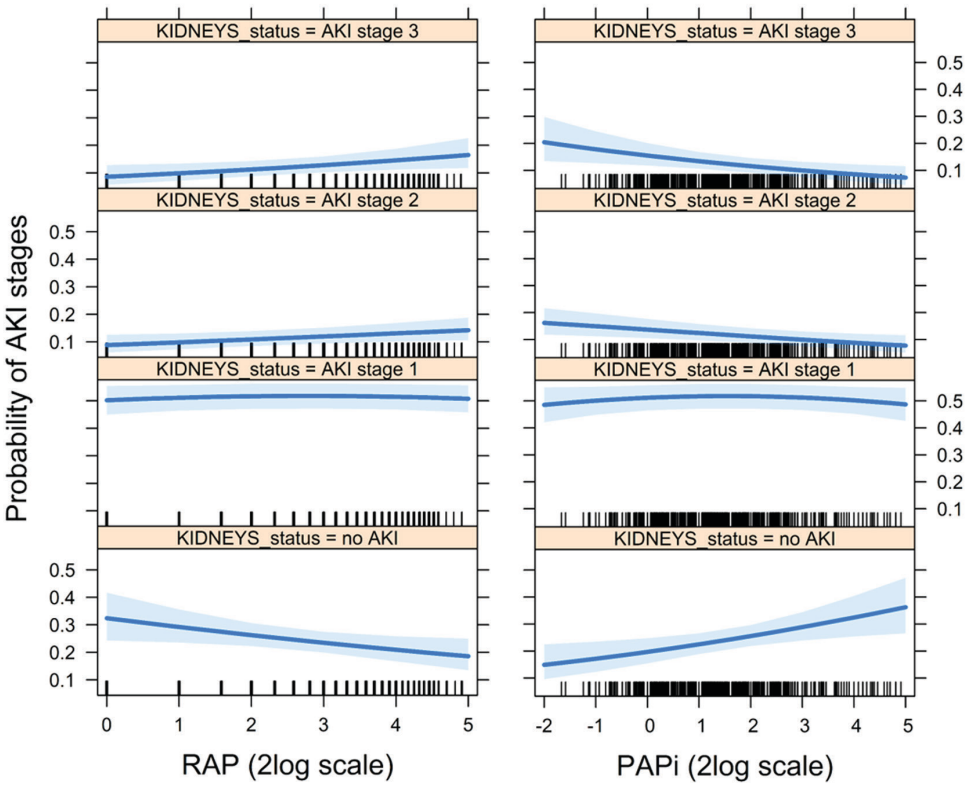


FIGURE S3 The relationships between preoperative RAP and PAPi values and the probabilities of different postoperative AKI stages early after heart transplantation. The X-axis on the left panel depicts the right atrial pressure (RAP) values on the ^{2}log scale, whereas the X-axis on the right panel depicts pulmonary artery pulsatility index (PAPi) values on the ^{2}log scale. Probabilities of each acute kidney injury (AKI) stage are presented in the Y-axis in descending order from AKI stage 3 to no AKI. The figure shows that probabilities of AKI stages 2 and 3 are increasing with higher RAP and lower PAPi values, while the corresponding probabilities for no AKI are decreasing.

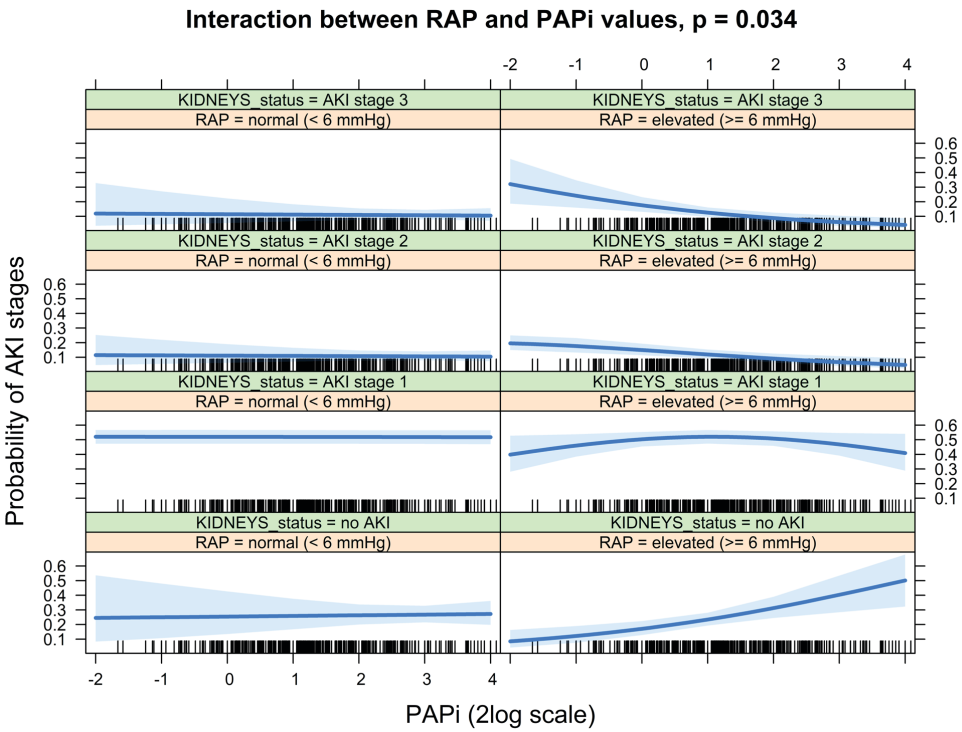
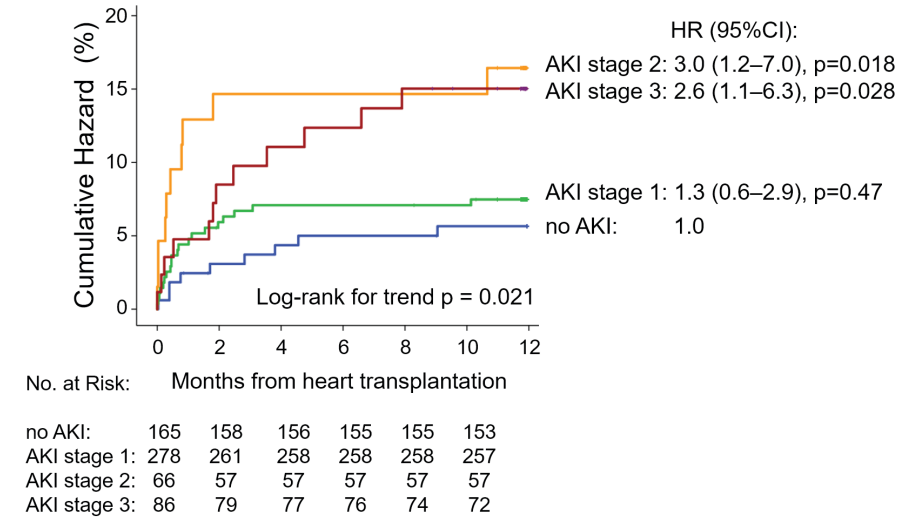


FIGURE S4 Graphical display of the interaction effect between preoperative RAP and PAPi on the probabilities of different postoperative AKI stages early after heart transplantation. The X-axis on the left panel depicts the pulmonary artery pulsatility index (PAPi) values on the ^{2}log scale in patients with normal right atrial pressure (RAP <6 mmHg), whereas the X-axis on the right panel depicts PAPi values in patients with elevated RAP (≥6 mmHg). Probabilities of each acute kidney injury (AKI) stage are presented in the Y-axis in descending order from AKI stage 3 to no AKI. The figure shows that probabilities of AKI stages 2 and 3 are markedly increasing with lower PAPi values in patients with elevated RAP, which is not the case in patients who had RAP within the reference range (p-interaction=0.034).

The following table displays the number of patients per category:

Number of patients	**Right atrial pressure**		
Kidneys status	< 6 mmHg	≥ 6 mmHg	Total
AKI stage 3	29	57	86
AKI stage 2	24	42	66
AKI stage 1	131	147	278
no AKI	71	94	165
Total	255	340	595



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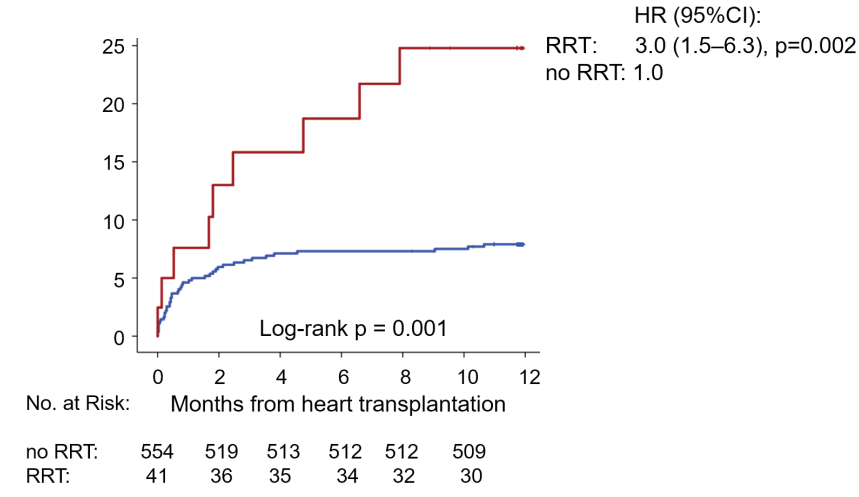

B

FIGURE S5 Kaplan-Meier curves for time to death during the first year after heart transplantation. A. Survival distribution analysis stratified by AKI stage with crude hazard ratios (95% confidence intervals) relative to no AKI. **B.** Analysis stratified by requirements for RRT during the first month after heart transplantation.

CHAPTER 13

Renal function and anemia in relation to short- and long-term mortality among patients with acute heart failure in the period 1985-2008

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ABSTRACT

Background

We investigated the prognostic value of renal dysfunction and anemia separately and in combination, on mortality in AHF patients. Furthermore, we examined whether the improvement in prognosis was comparable between patients with and without renal dysfunction.

Methods

This prospective registry includes 1783 patients admitted to the (Intensive) Coronary Care Unit for acute HF in the period of 1985-2008.

Results

In patients without renal dysfunction, anemia was associated with increased 30-day mortality (HR 2.91 [95% CI: 1.69–5.00]), but not with 10-year outcome (HR 1.13 [95% CI: 0.93-1.37]). On the contrary, anemia was found to influence prognosis in patients with renal dysfunction, both at 30 days (HR 1.93 [95% CI 1.33-2.80]) and at 10 years (HR 1.27 [95% CI 1.10-1.47]). Over time, the 10-year survival rate improved in patients with preserved renal function (HR 0.73 [95% CI 0.55-0.97]), but not in patients with renal dysfunction.

Conclusions

The long-term prognosis of acute HF patients with a preserved renal function was found to have improved significantly. However, the prognosis of patients with renal dysfunction did not change. Anemia was a strong predictor of short-term mortality in all patients. In patients with renal dysfunction, anemia was also associated with impaired long-term prognosis.

INTRODUCTION

Acute heart failure (HF) is commonly accompanied by various non-cardiovascular comorbidities. Renal dysfunction is among one of the most common although its exact prevalence has varied between studies.[1,2] Renal dysfunction in acute HF is associated with various adverse outcomes: longer hospital stay, higher re-hospitalization rate, and higher mortality.[1,2] Of note, the follow-up period in most of these studies is restricted to only 1 year after the initial hospitalization.

In the last decades, an improvement in long-term outcome has been observed among patients with acute HF in several cohorts.[3-5] New therapeutic options and an increased understanding of the pathophysiology of HF are most likely responsible for this trend. Importantly, renal dysfunction is a (relative) contraindication for some of the new therapeutic modalities.[6] As of yet, it has not been established whether the improvement in prognosis over time of patients with acute HF is modified by the presence of renal dysfunction.

Anemia is another important and common comorbidity in patients with acute HF, with a prevalence up to almost 60%.[7-12] There is conflicting data regarding the prognostic impact of anemia in patients with acute HF.[10-13] Moreover, the combination of HF, renal dysfunction and anemia carries an incremental negative prognostic impact in patients with chronic HF.[14] However, the additive prognostic value of anemia in patients with acute HF with and without renal dysfunction remains scarce.

Therefore, the aims of the present study were (1) to examine the impact of renal function on short- and long-term mortality of patients with acute HF, (2) to determine whether the improvement in prognosis of patients with acute HF and renal impairment was comparable to that of patients with normal renal function, and (3) to study the impact of anemia, alone or in combination with renal dysfunction, on mortality of patients with acute HF.

MATERIALS AND METHODS

Study population

This prospective registry was carried out among patients who were admitted with acute HF at the Intensive Coronary Care Unit (ICCU) in Erasmus MC, Rotterdam, the Netherlands during the period from 1985 until 2008. The study design and inclusion have been described previously.[5] Briefly, consecutive patients aged 18 years and older were included when they were diagnosed with acute HF or cardiogenic shock at admission. Both patients with de novo HF and patients with worsening symptoms of

chronic HF were included. Patients could only contribute once to the database, and if patients were admitted more than once with acute HF during the inclusion period, only the first admission was included for analyses. This was a prospective cohort registry. During the enrolment of the patients, approval from the local research ethics committee to conduct this study was not required. The study was conducted according to the Declaration of Helsinki.[15]

Study Endpoints

The 30-day mortality, 1-year mortality and 10-year mortality were the main outcome measures. Heart transplantation and left ventricular assist device implantation were considered as equivalent to mortality. Survival status was assessed using the Municipal Civil Registries in January 2017 and was available for 98% of the included patients.

Variables and definitions

Baseline variables were derived from patient records and discharge letters. We collected the following variables: age, gender, Body Mass Index (BMI), cardiac history, etiology of HF, left ventricular ejection fraction (LVEF) and treatment at the ICCU. Furthermore, the results of the following laboratory tests were collected: sodium (mmol/L), potassium (mmol/L), creatinine (μmol/L), urea (mmol/L) and hemoglobin (mmol/L).

Diabetes mellitus was considered to be present when patients received antidiabetic therapy. The LVEF was classified into the following qualitative categories: good, moderate and poor. If quantitative outcome for the LVEF was used, we applied the following cut-offs: >45%, 30-44% and <30% for good, moderate and poor LVEF, respectively.[5] The etiology of HF was categorized into ischemic cause versus non-ischemic cause of HF. For all laboratory tests, the first measured value during hospitalization was taken into account. The estimated glomerular filtration rate (eGFR) was estimated by using the Modification of Diet in Renal Disease (MDRD) equation for serum creatinine (μmol/L): $eGFR = 30849 \times \text{serum creatinine}^{-1.154} \times \text{age}^{-0.203} \times 0.742$ (if female) [eGFR in mL/min/1.73 m^2].[16] In line with the most recent HF guideline of the European Society of Cardiology,[6] a renal function was categorized as follows: preserved renal function: eGFR ≥60 mL/min/1.73m^2; moderately impaired renal function eGFR 30-59 mL/min/1.73m^2; severely impaired renal function eGFR <30 mL/min/1.73m^2. We used the definition of the World Health Organization to define anemia: hemoglobin <7.5 mmol/L in women and <8.2 mmol/L in men. Hyponatremia was defined as a serum sodium level ≤135 mmol/L. For the definition of hypo- and hyperkalemia the following cut-off values were applied: serum potassium <3.5 mmol/L and >5.0 mmol/L, respectively.

Statistical analysis

Categorical variables are presented as frequencies and percentages. The χ^2 test and the Fisher-Freeman-Halton exact test were used to compare categorical variables. Normally distributed, continuous data are presented as mean values with standard deviation and were compared using the one-way ANOVA. Continuous data that were not normally distributed are presented as median and interquartile range (IQR). The Mann-Whitney U test or the Kruskal-Wallis H test was used to compare these data.

Since data for LVEF and etiology were incomplete for, respectively, 28% and 12% of the patients, multiple imputations were performed by using baseline characteristics as predictors. Pooled means are given for LVEF and etiology.

The Kaplan-Meier method was used for presenting the cumulative mortality curves and they were compared using the log-rank test. Secondary analyses were carried out among the 30-day survivors. Logistic regression for 30-day mortality and the Cox proportional hazard method for long-term mortality were applied in order to examine the independent association between renal function and mortality, as well as between anemia and mortality. Adjustments were made for age, gender, history of HF, diabetes, hypertension, etiology of HF, atrial fibrillation at admission, LVEF, renal function and anemia.

All tests were two-tailed and p-values <0.05 were considered statistically significant. Results of logistic regression and the Cox proportional hazard model were reported as odds ratios (ORs) and hazard ratios (HRs), respectively, with their corresponding 95% confidence interval (95% CI). All statistical analyses were carried out using SPSS software (SPSS 21.0, IBM Corp., Armonk, NY, USA).

RESULTS

Baseline characteristics

In total, 1810 patients were admitted with acute HF in the period 1985-2008. Of these, 1783 (99%) patients had at least one creatinine measurement and they constitute the present study population. Over half of the patients were found to have renal dysfunction, which was severely impaired in 18% of patients. The proportion of patients with severe renal impairment remained stable over time, while the number of patients with preserved renal function increased and moderately impaired renal function became less prevalent ($p<0.001$; Figure 1).

Compared to patients with renal dysfunction, patients with preserved renal function were on average 6 years younger (Table 1). In addition, they less often had prior

myocardial infarction and coronary revascularization. With decreasing renal function, the prevalence of prior HF, diabetes and hypertension increased. Hyponatremia was also more common in patients with renal dysfunction, as was anemia.

Regarding therapy, patients with renal impairment were more frequently treated with intubation and mechanical ventilation, mechanical circulatory support and inotropic agents (Table 1). Moreover, the degree of renal impairment was associated with lower in-hospital usage of beta-blockers, ACE-inhibitors and diuretics.

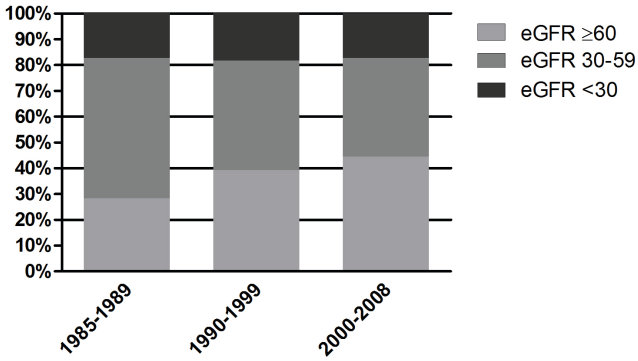

FIGURE 1 Distribution of the study population according to the renal function and the admission period. eGFR, estimated glomerular filtration rate in mL/min/1.73m^2

TABLE 1 Baseline characteristics and therapy according to renal function.

	eGFR ≥60	eGFR 30-59	eGFR <30	p-value*
No. of patients	688 (39%)	778 (44%)	317 (18%)	
Age, years (mean±SD)	59.7 ± 16.3	66.1 ± 13.2	65.9 ± 12.9	<0.001
Male gender	458 (67%)	475 (61%)	201 (63%)	0.09
BMI, kg/m^2 (mean±SD)	25.4 ± 5.2	24.9 ± 4.8	25.0 ± 4.7	0.57
Medical history				
Myocardial infarction	237 (34%)	347 (45%)	120 (38%)	<0.001
Coronary revascularization†	124 (18%)	187 (24%)	75 (24%)	0.01
Heart surgery (not CABG)	111 (16%)	87 (11%)	36 (11%)	0.01
Heart transplantation	2 (0.3%)	1 (0.1%)	6 (2%)	0.002
Waiting for heart transplantation	16 (2.3%)	11 (1.4%)	8 (2.5%)	0.33
Heart failure	300 (44%)	390 (50%)	188 (59%)	<0.001
Rhythm- or conduction disorder	157 (23%)	210 (27%)	73 (23%)	0.14
Diabetes	132 (19%)	168 (22%)	81 (26%)	0.07
Hypertension	194 (28%)	257 (33%)	133 (42%)	<0.001
Heart failure				
Etiology of heart failure				<0.05

continued

	eGFR ≥60	eGFR 30-59	eGFR <30	p-value*
Ischemic origin	302 (44%)	392 (50%)	140 (44%)	
Non-ischemic origin	386 (56%)	386 (50%)	177 (56%)	
Atrial fibrillation at admission	159 (23%)	178 (23%)	49 (16%)	0.01
Left ventricular ejection fraction				<0.05
Good	199 (29%)	225 (29%)	91 (29%)	
Moderate	187 (27%)	156 (20%)	76 (24%)	
Poor	302 (44%)	396 (51%)	149 (47%)	
Laboratory values				
Sodium (mean±SD)	137 ± 5	137 ± 6	135 ± 6	<0.001
Potassium (mean±SD)	4.0 ± 0.6	4.2 ± 0.7	4.6 ± 0.9	<0.001
Urea (median, IQR)	7.2 (5.7-9.3)	10.6 (8.2-14.4)	23.5 (17.5-30.8)	<0.001
eGFR (median, IQR)	75 (66-89)	47 (39-53)	20 (14-25)	<0.001
Creatinine (median, IQR)	80 (71-96)	123 (109-142)	258 (215-346)	<0.001
Hemoglobin	8.3 ± 1.3	8.1 ± 1.4	6.9 ± 1.5	<0.001
Hyponatremia	221 (32%)	224 (29%)	151 (48%)	<0.001
Hypokalemia	106 (15%)	98 (13%)	23 (7%)	<0.001
Hyperkalemia	38 (6%)	81 (10%)	85 (27%)	<0.001
Anemia	262 (38%)	334 (43%)	244 (77%)	<0.001
Therapy during ICCU hospitalization				
Intubation	69 (10%)	117 (15%)	57 (18%)	0.001
Resuscitation	19 (3%)	36 (5%)	15 (5%)	0.13
Mechanical circulatory support‡	34 (5%)	41 (5%)	29 (9%)	0.02
Inotropics	196 (29%)	253 (33%)	123 (39%)	0.01
Beta-blocker	146 (21%)	111 (14%)	47 (15%)	0.001
Antiarrhythmics	115 (17%)	154 (20%)	45 (14%)	0.06
Calcium antagonist	77 (11%)	102 (13%)	72 (23%)	<0.001
Digitalis	300 (44%)	347 (45%)	87 (27%)	<0.001
ACE-inhibitor or ARB	422 (61%)	430 (55%)	113 (36%)	<0.001
Diuretics	640 (93%)	718 (92%)	257 (81%)	<0.001
Nitrates	234 (34%)	289 (37%)	121 (38%)	0.24
Nitroprusside	46 (7%)	74 (10%)	39 (12%)	0.01
Antiplatelet agents	200 (29%)	189 (24%)	71 (22%)	0.04
Oral anticoagulant	351 (51%)	406 (52%)	136 (43%)	0.02

ACE, Angiotensin-converting enzyme; ARB, Angiotensin receptor blocker; BMI, Body Mass Index; CABG, coronary artery bypass graft; eGFR, estimated glomerular filtration rate; ICCU, intensive cardiac care unit; IQR, interquartile range; *p for any difference; †Percutaneous coronary intervention and/or CABG; ‡Intra-aortic balloon pump and/or left ventricular assist device and/or extracorporeal membrane oxygenation.

Renal dysfunction and mortality

The median survival of patients with a severely impaired, moderately impaired and preserved renal function was 1.0, 2.1 and 4.4 years, respectively. The impact of renal function on outcome is shown in Figure 2 and Table 2.

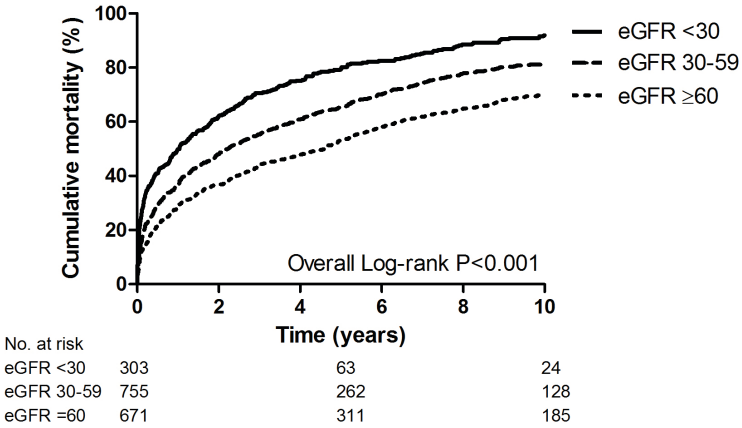

FIGURE 2 Survival curve of patients with acute heart failure according to the renal function. eGFR, estimated glomerular filtration rate in mL/min/1.73 m^2

TABLE 2 Mortality at different follow-up moments according to renal function.

	Mortality rate	Univariable analysis*	Multivariable analysis*
30 days			
eGFR ≥60	10%	Reference	Reference
eGFR 30-59	14%	1.51 (1.10-2.08)	1.50 (1.06-2.11)
eGFR <30	24%	2.85 (1.99-4.08)	2.32 (1.55-3.47)
1 year			
eGFR ≥60	28%	Reference	Reference
eGFR 30-59	36%	1.41 (1.17-1.69)	1.34 (1.11-1.62)
eGFR <30	50%	2.21 (1.79-2.73)	1.81 (1.44-2.28)
10 years			
eGFR ≥60	69%	Reference	Reference
eGFR 30-59	81%	1.42 (1.33-1.51)	1.24 (1.09-1.40)
eGFR <30	92%	2.14 (1.99-2.31)	1.68 (1.43-1.96)

*Odds ratio with 95% confidence interval (CI) for 30-day mortality, hazard ratio with 95% CI for 1-year and 10-year mortality; eGFR, estimated glomerular filtration rate in mL/min/1.73 m^2. Adjustments were made for age, gender, history of HF, diabetes, hypertension, etiology of HF, atrial fibrillation at admission, LVEF, renal function and anemia.

Patients with a severely impaired renal function had the worst prognosis both at short- and long-term. These findings remained unchanged after multivariable adjustment for other prognostic factors. Although the influence of renal function on prognosis became less prominent with longer duration of follow-up, renal function still remained a strong predictor of mortality.

Over time, the 10-year survival rate of patients with a preserved renal function improved significantly, both unadjusted (HR 0.70 [95% CI 0.61-0.81] for most recent period versus first period) and after adjustment for confounding variables (adjusted HR 0.73 [95% CI 0.55-0.97]; Figure 3A). This improvement was more pronounced among the 30-day survivors (adjusted HR 0.65 [95% CI 0.48-0.88]; Figure 3B). In contrast, this pattern was not present in patients with renal dysfunction. Consequently, the prognosis of these patients did not improve over time.

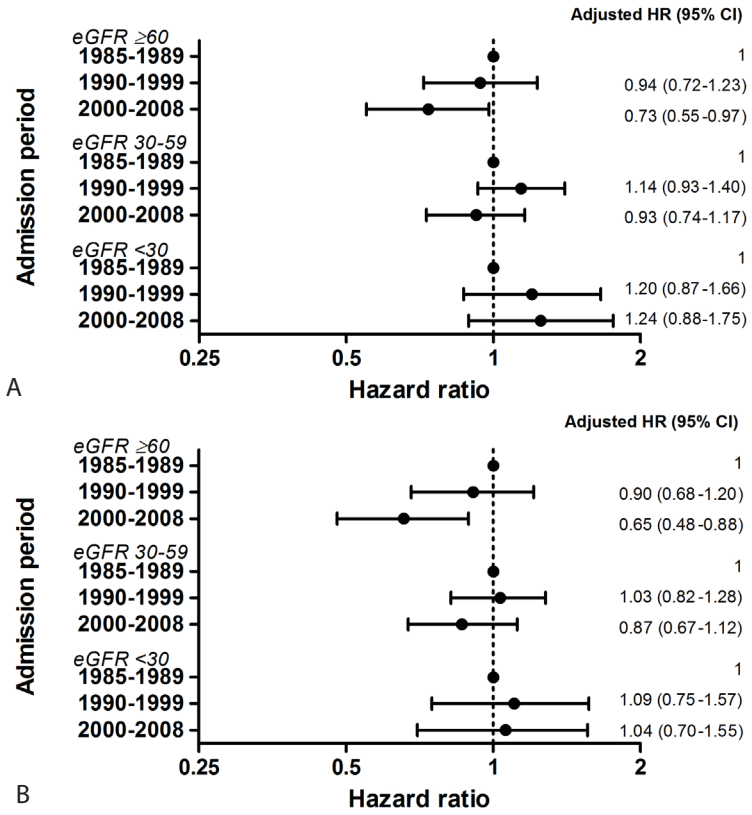

FIGURE 3 Mortality over time among (A) the total population and (B) the 30-day survivors of patients with acute heart failure. Results were divided into three groups according to the renal function. CI, confidence interval; eGFR, estimated glomerular filtration rate in mL/min/1.73 m^2; HR, hazard ratio.

Anemia and mortality

Almost 50% of the patients were found to have anemia. The characteristics of these patients differed in some aspects from those without anemia (Table 3). Anemic patients more frequently had previous HF and atrial fibrillation at admission. Importantly, they more often had impaired renal function.

The prognosis of patients with anemia was worse than of patients without anemia (Figure 4). After adjustment for confounders, anemia remained significantly associated with increased 30-day, 1-year and 10-year mortality (HR 2.23 [95% CI 1.64-3.03], HR 1.58 [95% CI 1.33-1.87] and HR 1.24 [1.11-1.39], respectively; Table 4).

TABLE 3 Baseline data and therapy of patients with and without anemia.

	Anemia +	Anemia -	p-value
No. of patients	850 (48%)	919 (52%)	
Age, years (mean±SD)	63.1 ± 14.5	64.1 ± 15.0	0.15
Male gender	565 (67%)	560 (61%)	0.02
BMI, kg/m^2 (mean±SD)	24.8 ± 4.8	25.4 ± 5.2	0.20
Medical history			
Myocardial infarction	336 (40%)	362 (39%)	0.95
Coronary revascularization*	199 (23%)	183 (20%)	0.07
Heart surgery (not CABG)	131 (15%)	102 (11%)	0.01
Heart transplantation	8 (0.9%)	1 (0.1%)	0.02
Waiting for heart transplantation	22 (2.6%)	12 (1.3%)	0.05
Heart failure	440 (52%)	425 (46%)	0.02
Rhythm- or conduction disorder	215 (25%)	218 (24%)	0.44
Diabetes	199 (23%)	181 (20%)	0.06
Hypertension	271 (32%)	308 (34%)	0.47
Heart failure			
Etiology of heart failure			>0.05
Ischemic origin	387 (45%)	438 (48%)	
Non-ischemic origin	463 (55%)	481 (52%)	
Atrial fibrillation at admission	141 (17%)	243 (26%)	<0.001
Left ventricular ejection fraction			>0.05

continued

	Anemia +	Anemia -	p-value
Good	260 (31%)	250 (27%)	
Moderate	192 (23%)	227 (25%)	
Poor	399 (47%)	442 (48%)	
Laboratory values			
Sodium (mean±SD)	136 ± 6	138 ± 5	<0.001
Potassium (mean±SD)	4.3 ± 0.8	4.1 ± 0.7	0.001
Urea (median, IQR)	12.6 (8.3-20.4)	8.4 (6.6-11.6)	<0.001
eGFR (median, IQR)	47 (26-64)	57 (43-73)	<0.001
Creatinine (median, IQR)	123 (94-200)	102 (82-130)	<0.001
Hemoglobin (mean±SD)	6.7 ± 0.9	9.0 ± 0.8	<0.001
Hyponatremia	359 (42%)	233 (25%)	<0.001
Hypokalemia	100 (12%)	125 (14%)	0.28
Hyperkalemia	122 (14%)	85 (9%)	0.001
Therapy during ICCU hospitalization			
Intubation	151 (18%)	92 (10%)	<0.001
Resuscitation	36 (4%)	34 (4%)	0.56
Mechanical circulatory support†	80 (9%)	23 (3%)	<0.001
Inotropics	329 (39%)	238 (26%)	<0.001
Beta-blocker	128 (15%)	174 (19%)	0.03
Antiarrhythmics	143 (17%)	165 (18%)	0.53
Calcium antagonist	130 (15%)	123 (13%)	0.25
Digitalis	305 (36%)	419 (46%)	<0.001
ACE-inhibitor or ARB	417 (49%)	540 (59%)	<0.001
Diuretics	747 (88%)	854 (93%)	<0.001
Nitrates	295 (35%)	342 (37%)	0.27
Nitroprusside	73 (9%)	86 (9%)	0.57
Antiplatelet agents	238 (28%)	224 (24%)	0.08
Oral anticoagulant	383 (45%)	497 (54%)	<0.001

ACE, Angiotensin-converting enzyme; ARB, Angiotensin receptor blocker; BMI, Body Mass Index; CABG, coronary artery bypass graft; eGFR, estimated glomerular filtration rate; ICCU, intensive cardiac care unit; IQR, interquartile range; *Percutaneous coronary intervention and/or CABG; †Intra-aortic balloon pump and/or left ventricular assist device and/or extracorporeal membrane oxygenation.

TABLE 4 **Mortality at different follow-up moments according to the presence of anemia.**

	Mortality rate	Univariable analysis*	Multivariable analysis*
30 days			
No anemia	9%	Reference	Reference
Anemia	20%	2.55 (1.92-3.38)	2.23 (1.64-3.03)
1 year			
No anemia	28%	Reference	Reference
Anemia	43%	1.75 (1.49-2.05)	1.58 (1.33-1.87)
10 years			
No anemia	75%	Reference	Reference
Anemia	83%	1.35 (1.28-1.43)	1.24 (1.11-1.39)

* Odds ratio with 95% confidence interval (CI) for 30-day mortality, hazard ratio with 95% CI for 1-year and 10-year mortality. Adjustments were made for age, gender, history of HF, diabetes, hypertension, etiology of HF, atrial fibrillation at admission, LVEF, renal function and anemia.

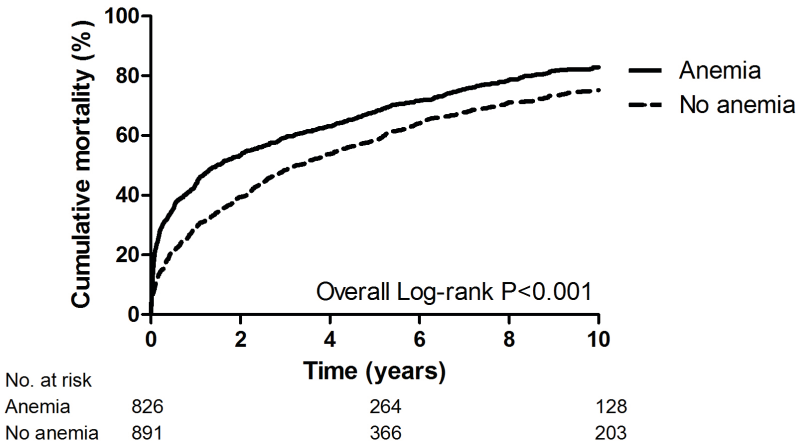

FIGURE 4 **Survival curve of acute heart failure patients with and without anemia.**

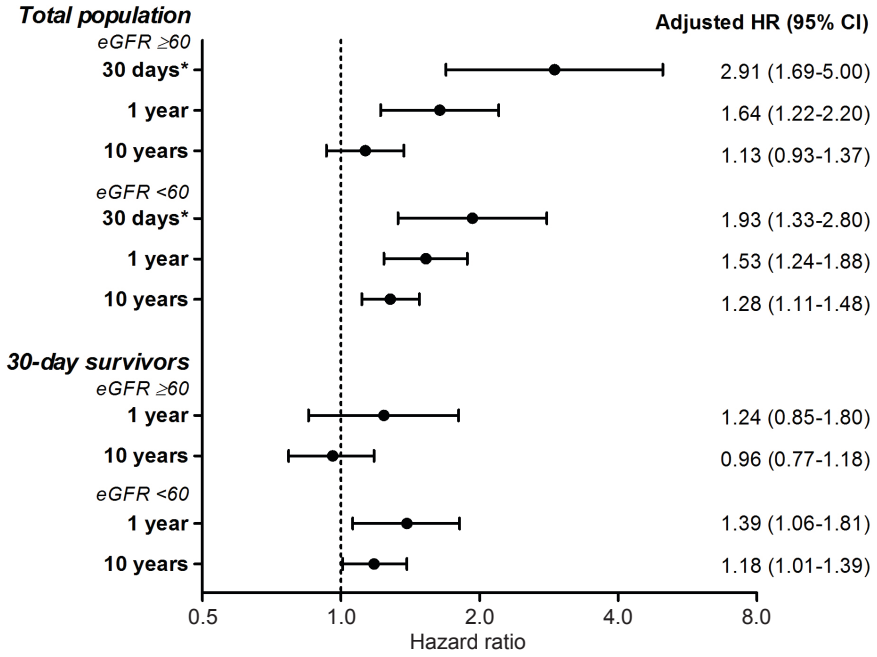

FIGURE 5 Prognostic impact of anemia at different follow-up moments in the total population and 30-day survivors. Analyses were separately done for renal impairment whether or not. CI, confidence interval; eGFR, estimated glomerular filtration rate in mL/min/1.73 m^2; HR, hazard ratio; *outcome at 30 days was reported as odds ratio with 95% CI.

Since anemia was a predictor of poor outcome in the total population of acute HF patients, we separately analyzed whether anemia had incremental prognostic value independent from renal dysfunction (Figure 5). Among patients with a preserved renal function, anemia proved to be a strong predictor for 30-day mortality, but its prognostic value decreased with longer duration of follow-up. In contrast, anemia was associated with worse outcome both during short- and long-term follow-up among patients with renal dysfunction. This relationship persisted after the exclusion of patients who died within 30 days after admission.

DISCUSSION

In this prospective registry of patients with acute HF, we found that renal dysfunction was a strong predictor for poor outcome up to 10 years following initial hospitalization. Importantly, this study is the first to show that patients with acute HF and an impaired renal function had no improvement in prognosis that occurred in the last three decades. This contrasts findings in patients with a preserved renal

function. Furthermore, we found that the prognostic impact of anemia was dependent on the presence of renal function. Anemia had no impact on the long-term prognosis of patients with a preserved renal function. On the other hand, anemia was associated with impaired prognosis among patients with renal dysfunction.

Renal dysfunction and mortality

Renal dysfunction proved to be a strong predictor of a poor outcome: the poorer the renal function, the poorer the prognosis. Among studies that demonstrated the adverse association between renal dysfunction and poor survival,[1,2] most only used a short follow-up period, usually up to 1 year after hospitalization. Our results support and extend these findings by demonstrating that renal dysfunction continued to be a strong predictor for long-term mortality (i.e., 10 years).

It is generally assumed that the new therapeutic options for the treatment of HF developed during the last decades are responsible for the prognostic improvement in the total population of patients acute HF. Our finding that only patients with a normal renal function experienced an improved long-term prognosis in the most recently study period is novel. This contrasts with the findings currently obtained among patients with renal dysfunction. Their prognosis remained stable over time. So far, the temporal trends in prognosis have not been studied separately for patients with and without renal dysfunction. Two potential mechanisms may explain this finding. First, some of the new therapeutics, like ACE inhibitors, ARBs and MRAs, that are considered to be responsible for the prognostic improvement of patients with HF over the last decades, interact with the renal function.[6] Therefore, it is plausible that patients with renal dysfunction were less frequently treated with these drugs and that, in case they were treated, the optimal dose was not achieved. Indeed, we found that ACE inhibitors were less frequently prescribed during admission in patients with renal dysfunction. Although data on medical therapy during follow-up were not included in this registry, it can be assumed that this pattern of prescription continued after discharge. Another possible explanation for the disparity in temporal trends between patients with and without renal dysfunction may be the grade of their illness. Patients with renal dysfunction had more comorbidities and were more frequently treated with intubation, mechanical circulatory support and inotropics than patients with preserved renal function. This suggests that patients with renal dysfunction were more critically ill as compared to those with a preserved renal function, and they might thus experience a more progressive course of their disease and, therefore, a poorer prognosis.

Anemia and mortality

The second result of our study was the finding that anemia was associated with both an impaired short- and long-term prognosis among patients with acute HF. The relation between anemia and adverse outcome in patients with acute HF has been published previously, although the data are not consistent.[10-13] Two studies that did not report anemia to be a prognosticator of poor outcome had study populations with quite different characteristics than ours.[10,13]

When we studied the prognostic value of anemia in more detail, we found that anemia was an independent predictor of short-term mortality in all patients, irrespective of renal function. However, while anemia also was independently associated with an impaired outcome during long-term follow-up in patients with renal dysfunction, its presence had no incremental long-term prognostic impact in patients with a preserved renal function. The reasons for this difference are not totally clear. A possible explanation may be the actual cause of the anemia. However, as we were not able to assess the exact etiology of the anemia, the following hypothesis should be studied further in the future.

Anemia in patients with HF is well known, and has been attributed to multiple factors including iron deficiency, renal dysfunction, HF as a chronic disease and hemodilution.[14] The iron status was not assessed in our patients so we cannot make any conclusions as whether there was a difference in iron status between patients with and without renal dysfunction. The fact that anemia was associated with impaired long-term outcome in patients with renal dysfunction but not in patients with a preserved renal function might be due to the fact that patients with renal dysfunction more frequently had 'true anemia'.

Hemodilution is one of the potential causes of anemia in patients with HF.[17] The causal factor in that case is a low hemoglobin level caused by an increased extracellular volume. When the extracellular volume decreases, for example by diuretic therapy, the hemoglobin level will increase and the patient will no longer be classified as having anemia. Therefore, in case of hemodilution anemia should be seen as a marker of fluid retention, just as sodium level. We hypothesize that hemodilution as the only cause of anemia was more frequent in patients without renal dysfunction than in those with renal dysfunction. Probably, patients with an impaired renal function had also anemia based on hemodilution but in addition, could also have suffered from 'true anemia'. There are several reasons for such a phenomenon. First, it is well known that renal failure is associated with anemia.[14] Second, in our study, chronic HF was more common among patients with renal dysfunction than among those without renal dysfunction. Since chronic HF has been associated with elevated plasma levels of cytokines,[18] chronic HF can cause anemia of chronic diseases. These

cytokines suppress the erythropoietic stem cells in the bone marrow and reduce the release of iron form the reticulo-endothelial system, resulting in anemia.[19]

The so-called cardiorenal anemia syndrome has not been investigated extensively in patients with acute HF. Investigators form the ATTEND registry also found anemia to be a strong predictor of in-hospital mortality both among patients with and without renal dysfunction.[20] Furthermore, their results with respect to the 1-year outcome were consistent with our data. In addition, these authors also showed that anemia had additive prognostic value for increased 1-year mortality only in the patients with renal dysfunction but not in those with a preserved renal function.[21] Because these investigators used anemia at discharge as predictor, and thus made hemodilution less likely as cause from anemia, this supports our hypothesis of 'true anemia' among patients with renal dysfunction. Our data provide new evidence on the very long-term prognosis of patients with acute HF since we found that anemia, even after 10 years of follow-up, continued to have additive prognostic value among patients with renal dysfunction.

The unique strength of our study is the duration of the follow-up of 10 years after the initial hospitalization. This enabled us to investigate the prognostic impact of renal dysfunction, anemia, as well as their interrelationship on short- en (very) long-term. Research covering three decades with such a long follow-up time is quite unique in this research field.

Study limitations

Despite these strengths, some limitations should be considered in the interpretation of the results of this study. Since our study was done in a tertiary referral hospital, external validity could have been affected. However, despite the fact that our hospital was a tertiary referral center, a significant part of our patients still were primary and secondary referrals. Therefore, our population consisted of patients within the whole, broad range of patients admitted with acute HF. Second, we were not able to identify the cause of anemia in all patients, nor were we always able to assess whether patients had chronic or acute renal dysfunction. Furthermore, while it has been suggested that changing hemoglobin and creatinine levels during admission may influence prognosis,[2,22] the design of our study did not allow us to assess trends in hemoglobin and creatinine levels. Finally, since we had no data on the ethnicity of our patients, we could not multiply for black race in the MDRD formula. Therefore, the eGFR that we employed might be an underestimation of the real renal function. However, such misclassification could have only led to underestimation of the effects observed.

CONCLUSION

We found renal dysfunction to be a strong predictor of both short- and long-term mortality among patients with acute HF. In addition, we established that the long-term prognosis of patients with a preserved renal function significantly improved over the last decades. However, in patients with renal dysfunction, the prognosis did not improve over the last decades. These findings emphasize the importance of renal dysfunction as comorbidity in patients with HF and underscore the need for new therapeutic modalities, especially for patients with renal dysfunction. Furthermore, we established anemia as a prognosticator of short-term mortality both among acute HF patients with and without renal dysfunction. Among patients with renal dysfunction, the presence of anemia was also associated with impaired long-term prognosis. Anemia did not influence the long-term prognosis of patients with preserved renal function. Further research should be undertaken to investigate the pathogenesis of the prognostic impact of anemia and renal dysfunction among patients with acute HF.

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CHAPTER 14

DISCUSSION

The understanding of the interactions between the heart and the kidneys is fundamental to achieve proper assessment and management of patients in whom both organ systems are affected. In this thesis, new findings on heart-kidney interactions are presented with particular focus on the temporal aspects of their relationship. For this purpose, we have studied several aspects of kidney functioning in a wide spectrum of at-risk populations such as patients with heart failure (HF) including those with acute HF, chronic HF, and end-stage HF, and patients with ischemic heart disease (IHD) at different stages of their disease.

The main objective of this work was to identify and quantify new signals along the heart-kidney axis that precede and relate to adverse clinical outcomes, but also to place these new findings in the context of improvement of a patient's risk assessment and management. The following summary addresses the main findings of this thesis and discusses their clinical perspectives and future directions.

SUMMARY

Part I Methodological concepts

The first part of this thesis focuses on two important methodological concepts within the current trends in clinical research. The first concept is dynamic prediction modeling using repeated-measures study designs to assess the dynamic nature of medical conditions and to derive personalized estimates of prognosis (**chapter 2**). Such study designs include repeated measurements of biological markers over the time-course of a disease, which enables us to perform valid inferences on disease dynamics and to assess patient prognosis. Yet, when analyzing repeatedly measured biological markers the question arises how to properly relate this

information to prognosis. To this end, we explain the joint modeling of repeatedly measured and time-to-event data within an individual patient to derive personalized prognosis using time-varying markers.

The second concept includes analysis of statistical interaction to assess whether the effect of a certain factor (i.e., exposure or intervention) on a certain outcome differs across different types of patients or whether a combined effect of the factors exceeds their effects considered separately (**chapter 3**). To this end, review studies on statistical interaction have demonstrated that it is studied in the majority of clinical studies. However, most studies still have difficulties to properly assess, interpret, and report this kind of analysis. Therefore, chapter 3 outlines the challenges associated with assessment, interpretation, and reporting of statistical interactions in clinical studies, as well as recommendations that, if adhered to, will increase the clarity and the completeness of future studies.

Part II The role of the kidneys in heart failure and beyond

The second part of this thesis focuses on the role of the kidneys in chronic heart failure (HF) using the unique study design of the *Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT)* study. The Bio-SHiFT study is a prospective cohort of 263 clinically stable patients with chronic HF recruited during their regular outpatient visits at Erasmus MC, in Rotterdam, and at Noordwest Ziekenhuisgroep, in Alkmaar, the Netherlands. Study follow-up visits were predefined by the study protocol, and scheduled every 3 months to a maximum follow-up duration of 30 months. At baseline and at each study follow-up visit, a medical evaluation was performed and both blood and urine samples were collected. This unique repeated-measures design allowed us to explore in detail temporal trajectories of many biomarkers during progression of chronic HF. These dynamic biomarker patterns were subsequently used to estimate a patient's risk of future adverse clinical outcomes. By doing so, a window of opportunity may be gained to timely modify the treatment before a future outcomes occurs.

Chapter 4 demonstrates that renal dysfunction is an indivisible component of the syndrome of HF, but its single assessment does not sufficiently reflect clinically silent progression of HF prior to adverse outcome. To our best knowledge, this study is the first to simultaneously assess glomerular and tubular function over time during several years of follow-up and to show that both renal compartments chronically deteriorate, but not in parallel, during progression of HF. The results demonstrate that patient-specific trajectories of glomerular indices (creatinine, es-

timated glomerular filtration rate [$eGFR_{Cr}$], and cystatin C) and tubular damage markers (urinary N-acetyl-beta-D-glucosaminidase [NAG] and kidney-injury-molecule [KIM]-1) predict adverse clinical outcomes independently of patients' clinical characteristics, pharmacological treatment, cardiac natriuretic peptides and troponins, and for tubular markers also independently of eGFR. In this context, both the levels of these renal markers and their slopes (i.e., rates of change) may be useful for dynamic risk profiling. Such dynamic risk profiling can enable physicians to better detect disease progression and to make individualized treatment decisions.

In **chapter 5**, we further explored decline of glomerular filtration (GD) and progressive tubular damage (PTD) during clinically silent progression of chronic HF. We found that if GD and PTD coexist during follow-up the clinical prognosis worsens. Yet, PTD carried poor prognosis even in the absence of GD. This is particularly important to note since in current clinical practice tubular markers are not routinely assessed, leaving the degree of PTD undetermined. However, our findings suggest that "renoprotective" treatment targeted at the tubules may be even more effective than treatment aiming at improving renal function in terms of eGFR. This issue will be further explored in chapter 12 where we investigated the temporal effects of HF medication adjustments on these biomarkers during patients' follow-up. Finally, patients' clinical profiles differed between PTD and GD, which also supports that these renal indices should be jointly assessed.

Chapter 6 describes the temporal profiles of new, emerging cardiorenal and pulmonary candidate biomarkers during clinically silent stages of chronic HF. In this regard, osteopontin (OPN), osteoprotegerin (OPG), trefoil factor-3 (TFF-3), and heparin-binding protein (HBP) strongly predicted adverse clinical outcomes in patients with chronic HF. The use of these markers may be clinically relevant as they may further refine estimation of a patient's prognosis, provide additional pathophysiological insights into HF, and may ultimately be useful for designing more effective strategies for biomarker (trajectory)-guided therapy.

Although it is known that multiple hormonal and metabolic alterations occur in chronic HF, the cardiometabolic biomarkers that reflect these alterations have been insufficiently explored. **Chapter 7** exclusively demonstrates that temporal trends of cardiometabolic biomarkers such as insulin-like binding protein (IGFBP)-1, -2, and -7, as well as adipokine fatty-acid binding protein-4 are strongly related to adverse clinical outcomes in patients with clinically stable HF. Their clinical role may be to assist in the care for HF patients by means of better phenotyping of the disease, but also their dynamical changes may be practically important to detect more aggressive forms of chronic HF.

Furthermore, **chapter 8** describes the prognostic value of dynamic profiles of fourteen cardiac remodeling candidate biomarkers during follow-up of patients with chronic HF including ST2, Gal-3, Gal-4, GDF-15, MMP-2, 3 and 9, TIMP-4, PLC, AP-N, CASP3, CTSD, CTSZ and CSTB. Their dynamic nature is important considering the dynamic nature of myocardial remodeling, which has a pivotal role in the progression of HF.

Altogether, our results suggest a promising role for repeatedly assessed, established and novel biomarkers in prognostication of patients with chronic HF. As a next step, well-organized clinical trials are needed to provide definite evidence if such established markers, as well as the novel biomarkers that are expected to emerge in near future, can be used for biomarker (trajectory)-guided therapy.

Part III Implications of renal function for ischemic heart disease

Not only does renal dysfunction play one of the key roles in the syndrome of HF, altered renal function is also a major determinant of cardiovascular outcome in patients with IHD. Therefore, part III focuses on the implications of renal function for patients with acute coronary syndrome (ACS) and for those with stable angina pectoris (SAP).

In **chapter 9**, we studied patients hospitalized with ACS enrolled in the *BIOMarker study to identify the Acute risk of a Coronary Syndrome (BIOMArCS),* which is a multi-centre prospective study conducted in 18 Dutch hospitals. Here, our aim was to describe the evolution of renal function from its initial change during ACS until stabilization; and to investigate the predictive value of serial renal assessments during the first year on the occurrence of clinical events, including recurrent ACS or death. Considering that existing studies have mostly investigated renal function only at a single time moment (e.g., at admission, a moment during in-hospital stay, or at discharge), its temporal patterns following and preceding ACS remain unclear. Knowing these temporal patterns may help us identify high-risk subgroups of patients with ACS. In this regard, we demonstrate that plasma cystatin C levels indicate disturbances in renal function earlier than creatinine or eGFR do during the initial ACS. We also show that disturbances in renal indices usually do not stabilize during hospitalization, which usually lasts up to 7 days, but on average stabilize two weeks after ACS. Finally, we show that during the first year of follow-up after ACS, cystatin C levels predict clinical events independently of GRACE risk score in patients with normal to moderately impaired renal function.

In **chapter 10**, we focus on the relations of a glomerular marker, plasma cys-

tatin C, and a tubular marker, plasma neutrophil gelatinase-associated lipocalin (NGAL), with coronary atherosclerosis and occurrence of 1-year major adverse cardiac events (MACE) within the *European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study*. The ATHEROREMO-IVUS study included patients who underwent coronary angiography for ACS or SAP and in whom intravascular ultrasound (IVUS) imaging of a non-culprit coronary artery was performed. Using virtual histology (VH)-IVUS, the extent and composition of coronary atherosclerosis were assessed and high-risk lesions were identified including thin-cap fibroatheroma (TCFA), lesions with plaque burden (PB)≥70%, and lesions with minimal luminal area ≤4.0 mm^2. In patients with normal renal function higher CysC levels were associated with fewer high-risk lesions such as TCFA and PB≥70%. However, in patients with mildly-to-moderately impaired kidneys these 'protective' effects of cystatin C were absent, and higher cystatin C levels predicted MACE. Conversly, NGAL did not show clear assciations with coronary atherosclerosis or MACE. These findings indicate that renal dysfunction modifes the relationship between plasma cystatin C and coronary atherosclerosis.

Part IV Lessons learned from clinical practice

The fourth part of this thesis investigates several aspects of current clinical practice in order to provide additional insights into heart-kidney interactions.

Chapter 11 describes the temporal effects of neurohormonal antagonists and loop diuretics on serially assessed cardiac, renal, and anti-inflammatory biomarkers, patient functional status, and occurrence of adverse clinical outcomes during outpatient follow-up of patients with chronic HF and reduced ejection fraction within the Bio-SHiFT study. Here, we found that decrease in dosage or withholding of angiotensin-converting enzyme (ACE)-inhibitors/angiotensin II receptor blockers (ARBs) solely based on glomerular function is not justified because of their beneficial cardiac, tubular and anti-inflammatory effects. To our best knowledge, our findings are the first to show the beneficial effects of ACE-inhibitors/ARBs on renal tubules in chronic HF. Finally, higher dosage and increase in dosage of loop diuretics during follow-up marked progression towards end-stage HF.

In patients undergoing heart transplantation (HTx), postoperative acute kidney injury (AKI) continues to be a frequent complication with poor prognosis (**chapter 12**). Although renal injury after heart transplantation has traditionally been attributed to impaired arterial perfusion and calcineurin inhibitors' nephrotoxicity, our results provide strong evidence for an independent relationship between preopera-

tive right-sided hemodynamics (lower pulmonary artery pulsatility index [PAPi] and higher right atrial pressure [RAP] values) and postoperative AKI severity. Altogether, these data suggest that preoperatively compromised right-sided venous pressures deserve clinical attention in the context of postoperative AKI and that preoperative PAPi and RAP values may be used as early AKI predictors.

In **chapter 13**, we explore our 23-year long registry data (from 1985 to 2008) on the short- and long-term clinical prognosis of patients hospitalized with acute HF. Our results demonstrate that long-term prognosis of patients in whom renal function is preserved has significantly improved in recent years. However, the long-term prognosis in those with renal dysfunction has remained impaired. We also found that the presence of anemia was associated with poor short-term prognosis in all patients. Finally, in patients with renal dysfunction, anemia was associated with impaired long-term prognosis.

MAIN CONCLUSIONS

This thesis suggests a promising role for dynamic prediction modeling using repeatedly assessed, established and novel biomarkers in prognostication of patients with chronic HF. Similarly, the statistical interaction analysis may help us to learn how to use an intervention most effectively, who would and who would not benefit, how patients' comorbidities influence the effect, and whether it would be harmful in specific subpopulations.

Although the failing heart affects both the glomerular and tubular compartments of the kidneys, the degree of damage in these renal compartments is usually not temporally coupled. Importantly, the deterioration of either glomerular or tubular compartment, and especially their simultaneous damage entail poor prognosis in chronic HF. During clinically silent progression of HF, the patient-specific evolutions of glomerular markers (plasma creatinine, eGFR and cystatin C) and tubular markers (urinary NAG and KIM-1) dynamically predict adverse clinical outcomes such as HF rehospitalizations and death. In a multi-organ syndrome such as HF, circulating biomarkers that reflect its multi-organ pathophysiology may be a valuable clinical tool, as these cellular signals precede cardiac decompensation and may provide early tissue- and organ-specific information. In this respect, we identified several new biomarkers that carry potential to further characterize the multi-organ pathophysiology of chronic HF, but may also help in monitoring disease progression during outpatient follow-up.

In patients with ACS, plasma CysC levels indicate deterioration of renal function earlier than creatinine-based indices do, and higher CysC levels contain prog-

nostic information for the recurrence of ACS and death during the first year after ACS in patients with mild-to-moderate renal dysfunction. Such renal dysfunction also modifies the relationship between CysC and the presence of VH-IVUS high-risk coronary lesions. These findings should not be neglected because such mild renal dysfunction usually does not require medical attention, yet the subtle differences captured by cystatin C appear to carry potential for improving patient risk stratification.

Decrease or withholding of ACE-inhibitors/ARBs solely based on glomerular function markers is not justified in stable patients with HF because of their beneficial cardiac, tubular and anti-inflammatory effects. In contrast, higher dosage and increase in dosage of loop diuretics during follow-up mark progression towards end-stage HF. In patients with end-stage HF, preoperative right-sided hemodynamic indices such as PAPi and RAP strongly predict severity of postoperative AKI, suggesting a key role for chronic renal venous congestion in renal injury early after heart transplantation.

CLINICAL PERSPECITVES AND FUTURE DIRECTIONS

This thesis describes additional insights into heart-kidney interactions that may enhance their early identification and their monitoring in order to improve risk prediction in patients in whom both organ systems are affected. Specifically, it demonstrates that temporal biomarker patterns assessed in individuals carry additional prognostic information on top of the traditional single (baseline) assessment approach. For physicians, it is also medically relevant to use all available information (baseline and follow-up) to accurately detect disease dynamics and to profile individual prognosis. Such dynamic prognostication could be integrated into clinical decision-making and could be particularly useful for tissue-specific targeting of therapies.

To accomplish this, we report the results of a large array of established and emerging biomarkers and cover various phases of biomarker research. These phases include initial proof of concept testing (i.e., assessing whether specific biomarkers significantly differ between patients with and without outcome), prospective validation (i.e., prediction of future outcomes in prospective cohort studies), and assessment of their incremental value to traditional risk predictors. As such, this thesis provides a solid basis for future studies to examine the clinical utility of the biomarkers investigated here, within a biomarker (trajectory)-guided treatment strategy. Altogether, we hope that the results of our work as reported in this thesis will contribute to reducing patients' mortality- and hospitalization rates, improving their quality of life, but also reducing healthcare costs.

NEDERLANDSE SAMENVATTING

Inzicht in de wisselwerking tussen hart en nieren is essentieel voor het adequaat beoordelen en behandelen van patiënten bij wie deze beide organen zijn aangedaan. In dit proefschrift worden nieuwe bevindingen besproken met betrekking tot de interactie tussen hart en nieren, met bijzondere aandacht voor het tijdsbeloop van de onderlinge relatie tussen deze orgaansystemen. We hebben hiervoor verschillende aspecten van de nierfunctie bestudeerd in een breed spectrum van risicopopulaties. Zo worden er in dit proefschrift patiënten met hartfalen (HF) besproken, waaronder patiënten met acuut HF, chronisch HF en eindstadium HF. Tevens wordt er aandacht besteed aan patiënten met ischemische hart ziekte (IHZ) in verschillende stadia van de ziekte.

Het hoofddoel van dit onderzoek was het identificeren en kwantificeren van nieuwe signalen op de hart-nier-as, die voorafgaan aan en betrekking hebben op ongunstige klinische uitkomsten. Eveneens was het voor ons van belang om deze nieuwe bevindingen te plaatsen in de context van verbetering van risicoschatting en behandelstrategie van de patiënt. De volgende samenvatting geeft een weergave van de belangrijkste bevindingen uit dit proefschrift en bespreekt de klinische perspectieven en toekomstige mogelijkheden.

Deel I Methodologische concepten

Het eerste deel van dit proefschrift focust op twee belangrijke methodologische concepten die passen binnen de huidige trend van klinisch onderzoek. Het eerste concept is dynamische voorspellingsmodellering, dat gebruik maakt van een onderzoeksopzet met herhaalde metingen. Het doel van dit model is om de dynamische aard van medische aandoeningen inzichtelijk te maken en om een gepersonaliseerde voorspelling van de prognose te verkrijgen (**hoofdstuk 2**). Dergelijke studieopzetten bevatten herhaalde metingen van biologische markers gedurende het beloop van een ziekte, hetgeen ons in staat stelt om conclusies te trekken over de dynamiek van de ziekte en om de prognose van de patiënt in te schatten. Bij het analyseren van herhaaldelijk gemeten biologische markers, dient de vraag zich aan hoe de veelvuldig gemeten markers op de juiste manier kunnen worden gerelateerd aan de prognose. Daarom gebruikten we “joint modeling”, waarbij rekening wordt gehouden met herhaalde (biomarker) metingen in een individuele patiënt, maar ook met verschillen tussen de patiënten in de database. Deze methode stelt ons in staat om de herhaalde (bio)marker metingen over de tijd te gebruiken om een gepersonaliseerde prognose in te schatten.

Het tweede methodologische concept omvat statistische interactie analyses. Deze analyses beoordelen of het effect van een bepaalde factor (bijvoorbeeld een blootstelling of een interventie) op een bepaald resultaat verschilt tussen verschillende typen patiënten of dat een gecombineerd effect van deze factoren groter is dan iedere factor afzonderlijk (**hoofdstuk 3**). Reviewonderzoek naar statistische interactie heeft aangetoond dat statistische interactie in veel klinische onderzoeken wordt bestudeerd. De meeste studies hebben echter moeite om dit soort analyses te beoordelen, te interpreteren en te rapporteren. Hoofdstuk 3 schetst daarom de uitdagingen die gepaard gaan met de beoordeling, interpretatie en rapportage van statistische interacties in klinische onderzoeken, waarbij ook aanbevelingen worden gegeven die de duidelijkheid en de volledigheid van toekomstige studies zullen vergroten.

Deel II De rol van de nieren in hartfalen en verder

Het tweede deel van dit proefschrift focust op de rol van de nieren in chronisch hartfalen doormiddel van de unieke studieopzet in de zogenoemde "*Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT)*" studie. De Bio-SHiFT studie is een prospectief cohort van 263 klinisch stabiele patiënten met chronisch HF. Deze patiënten zijn geïncludeerd tijdens hun reguliere polikliniek bezoek in het Erasmus MC, Rotterdam, of in de Noordwest Ziekenhuisgroep, Alkmaar, Nederland. Vervolgbezoeken voor de studie werden vooraf gedefinieerd in het onderzoeksprotocol, namelijk iedere 3 maanden, tot een maximale follow-up duur van 30 maanden. Zowel bij het eerste bezoek als bij alle vervolgbezoeken, werd een medische evaluatie uitgevoerd en werden er zowel bloed- als urinemonsters verzameld. Door deze unieke studieopzet van herhaalde metingen waren we in staat om tijdens het proces van chronisch HF het beloop van vele biomarkers te bestuderen. Deze dynamische patronen van de biomarkers werden vervolgens gebruikt om het risico van een patiënt te schatten op ongunstige klinische uitkomsten. Hierdoor ontstaat er een kans om de behandeling tijdig aan te passen, voordat deze negatieve ziekte-uitkomst optreedt.

Hoofdstuk 4 toont aan dat renale disfunctie onlosmakelijk verbonden is met het syndroom van HF. Echter, één afzonderlijke meting van de nier(dis)functie weerspiegelt niet voldoende de progressie van HF tijdens de klinisch ogenschijnlijk stabiele fase voorafgaand aan een ongunstige ziekte uitkomst. Voor zover wij weten is dit de eerste studie die zowel de glomerulaire als de tubulaire functie over een tijdsbestek van enkele jaren beoordeelt en daarmee aantoont dat tijdens het ziektebeloop van HF beide nier compartimenten chronisch verslechteren, maar dat

ze dit niet tegelijkertijd doen. De resultaten tonen aan dat het patiënt-specifieke beloop van glomerulaire indicatoren (creatinine, geschatte glomerulaire filtratiesnelheid [eGFRCr] en cystatin C) en markers van tubulaire schade (urinary N-acetyl-beta-D-glucosaminidase (NAG) en kidney-injury-molecule (KIM) -1) ongunstige klinische uitkomsten voorspellen. Deze bevindingen zijn onafhankelijk van de klinische kenmerken van een patiënt, farmacologische behandeling, cardiale natriuretische peptiden en troponinen en wat betreft tubulaire markers is de voorspellende waarde ook onafhankelijk van de eGFR. In deze context kunnen zowel de levels van de nier-markers, als ook hun helling (d.w.z. de snelheid van de verandering over de tijd) nuttig zijn voor dynamische risicoprofilering. Een dergelijke dynamische risicoprofilering kan de arts in staat stellen om progressie van de ziekte beter te detecteren en om geïndividualiseerde behandelbeslissingen te nemen.

In **hoofdstuk 5** hebben we de achteruitgang van glomerulaire filtratie (GD) en progressieve tubulaire schade (PTD) verder onderzocht tijdens progressie van chronisch HF, terwijl deze progressie klinisch nog niet waarneembaar was. We vonden dat als zowel GD als PTD gelijktijdig bestaan gedurende de follow-up, de klinische prognose slechter wordt. Alleen het hebben van PTD leidde ook tot een slechte prognose, zelfs bij afwezigheid van GD. Dit is met name belangrijk om op te merken, aangezien in de huidige klinische praktijk tubulaire markers niet routinematig worden bepaald, waardoor de mate van PTD onbekend blijft. Onze bevindingen suggereren echter dat "nier protectieve" behandelingen, gericht op de tubuli, zelfs effectiever kunnen zijn dan behandeling alleen gericht op het verbeteren van de nierfunctie in termen van de eGFR. Dit punt zal verder worden onderzocht in hoofdstuk 12, waar we de effecten van HF-medicijnaanpassingen over de tijd hebben onderzocht op deze biomarkers gemeten tijdens de follow-up. Ten slotte verschilden de klinische profielen van patiënten met PTD en GD, wat ook ondersteunt dat deze renale indicatoren gezamenlijk moeten worden beoordeeld.

Hoofdstuk 6 beschrijft de profielen van nieuwe, opkomende cardiorenale en pulmonale biomarker kandidaten over de tijd in de klinisch stabiele fase van chronisch HF. In dit opzicht voorspelden osteopontin (OPN), osteoprotegerin (OPG), trefoil factor-3 (TFF-3) en heparin-binding protein (HBP) sterk de ongunstige klinische uitkomsten bij patiënten met chronisch HF. Het gebruik van deze markers kan klinisch relevant zijn omdat ze de inschatting van de prognose van een patiënt kunnen verfijnen, extra pathofysiologische inzichten in HF kunnen geven en uiteindelijk nuttig kunnen zijn voor het opstellen van effectievere strategieën voor biomarker-geleide therapie.

Hoewel bekend is dat bij chronisch HF meerdere hormonale en metabole veranderingen optreden, zijn de cardiometabolische biomarkers die deze veranderin-

gen weerspiegelen onvoldoende onderzocht. **Hoofdstuk 7** laat uitsluitend zien dat trends van cardiometabolische biomarkers over de tijd zoals insulin-like binding protein (IGFBP)-1, -2 en -7, evenals adipokine fatty-acid binding protein-4 sterk gerelateerd zijn aan ongunstige klinische uitkomsten bij patiënten met klinisch stabiel HF. Deze biomarkers kunnen een klinische rol vervullen bij de zorg voor HF-patiënten door middel van betere fenotypering van de ziekte, maar ook de dynamische veranderingen kunnen van belang zijn om agressievere vormen van chronisch HF te detecteren.

Verder beschrijft **hoofdstuk 8** de prognostische waarde van 14 kandidaat biomarkers, die te maken met de remodelering van het hart, tijdens de follow-up van patiënten met chronisch HF. De prognostische waarden van de dynamische profielen van ST2, Gal-3, Gal-4, GDF-15, MMP-2, 3 en 9, TIMP -4, PLC, AP-N, CASP3, CTSD, CTSZ en CSTB worden hier beschreven. De dynamische aard van deze markers is belangrijk gezien de dynamische aard van myocardiale remodelering, wat een cruciale rol speelt in de progressie van HF.

Al met al suggereren onze resultaten een veelbelovende rol voor herhaaldelijk gemeten, klassieke maar ook nieuwe biomarkers bij de prognose van patiënten met chronisch HF. Als volgende stap zijn goed georganiseerde klinische onderzoeken nodig om definitief bewijs te leveren of de klassieke markers, evenals de nieuwe biomarkers die naar verwachting in de nabije toekomst zullen verschijnen, kunnen worden gebruikt voor biomarker (traject)-geleide therapie.

Deel III Implicaties van de nierfunctie voor ischemische hartaandoeningen

Niet alleen speelt renale disfunctie een sleutelrol in het syndroom van HF, een veranderding van de nierfunctie is ook een belangrijke bepalende factor voor cardiovasculaire uitkomsten bij patiënten met IHZ. Daarom richt deel III zich op de implicaties van de nierfunctie bij patiënten met acuut coronair syndroom (ACS) en patiënten met stabiele angina pectoris (SAP).

In **hoofdstuk 9** bestudeerden we patiënten die waren opgenomen in een ziekenhuis in verband met ACS en die deelnamen aan de "*BIOMarker study to identify the Acute risk of a Coronary Syndrome*" ofwel BIOMArCS studie. Dit is een prospectieve multicenter studie uitgevoerd in 18 Nederlandse ziekenhuizen. In dit hoofdstuk was ons doel om de evolutie van de nierfunctie te beschrijven vanaf de initiële verandering tijdens ACS tot stabilisatie. Tevens hebben we de voorspellende waarde van herhaalde nierfunctie metingen onderzocht tijdens het eerste jaar na een ACS op het optreden van klinische gebeurtenissen (waaronder opnieuw een

ACS of overlijden). In overweging nemende dat bestaande studies de nierfunctie meestal alleen op één enkel moment hebben onderzocht (bijvoorbeeld bij opname, een moment tijdens ziekenhuisverblijf of bij ontslag), blijven de temporele patronen na en voorafgaand aan ACS onduidelijk. Als we deze patronen over de tijd leren kennen kan dit ons helpen om risicovolle subgroepen van patiënten met ACS te identificeren. Vanuit dit oogpunt demonstreren we dat het niveau van plasma cystatin C eerder stoornissen in de nierfunctie aangeven dan creatinine of eGFR tijdens het initiële ACS. We laten ook zien dat stoornissen in de nier-indicatoren gewoonlijk niet stabiliseren tijdens de ziekenhuisopname, die meestal tot 7 dagen duurt, maar gemiddeld twee weken na het ACS stabiliseert. Tot slot laten we zien dat het niveau van plasma cystatin C, tijdens het eerste jaar van follow-up na een ACS, klinische verschijnselen kan voorspellen onafhankelijk van de GRACE-risicoscore bij patiënten met een normale tot matig verminderde nierfunctie.

In **hoofdstuk 10** concentreren we ons op de relatie van een glomerulaire marker, plasma cystatin C en een tubulaire marker, plasma neutrophil gelatinase-associated lipocalin (NGAL), met coronaire atherosclerose en het optreden ernstige cardiale aandoeningen (MACE) binnen 1 jaar in de "*European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound"(ATHEROREMO-IVUS*) studie. De ATHEROREMO-IVUS studie bestaat uit patiënten die coronaire angiografie ondergingen i.v.m. ACS of SAP en bij wie beeldvorming middels intravasculaire echografie (IVUS) van een "non-culprit" bloedvat van het hart werd uitgevoerd. Met behulp van virtuele histologie (VH)-IVUS werd de mate en samenstelling van coronaire atherosclerose vastgesteld en werden hoog-risico laesies geïdentificeerd, waaronder "thin-cap fibroatheroma" (TCFA), laesies met een "plaque burden" (PB) ≥70% en laesies met een minimale luminale oppervlakte ≤4.0 mm^2. Bij patiënten met een normale nierfunctie waren hogere cystatin C waarden geassocieerd met minder hoog-risico laesies, zoals TCFA en PB≥70%. Bij patiënten met een licht tot matig gestoorde nierfunctie waren deze 'beschermende' effecten van cystatin C afwezig en hogere niveaus van cystatin C voorspelden MACE. Omgekeerd toonde NGAL geen duidelijke associaties met coronaire atherosclerose of MACE. Deze bevindingen wijzen erop dat renale disfunctie de relatie tussen plasma cystatin C en coronaire atherosclerose modificeert.

Deel IV Lessen uit de klinische praktijk

Het vierde deel van dit proefschrift onderzoekt verschillende aspecten van de huidige klinische praktijk om aanvullende inzichten te bieden in de interacties tussen hart en nieren.

Hoofdstuk 11 beschrijft de effecten over de tijd van neurohormonale antagonisten en lisdiuretica op herhaaldelijk gemeten cardiale, renale en anti-inflammatoire biomarkers, functionele status van patiënten en het optreden van ongunstige klinische uitkomsten tijdens poliklinische follow-up van patiënten met chronisch HF en verminderde ejectiefractie in de Bio-SHiFT-studie. Hierin vonden we het verlagen van de dosering of het niet-geven van ACE-remmers/ARB's, uitsluitend gebaseerd op glomerulaire functie, niet gerechtvaardigd is vanwege de gunstige cardiale, tubulaire en ontstekingsremmende effecten die ze hebben. Voor zover wij weten zijn onze bevindingen de eerste die de gunstige effecten aantonen van ACE-remmers/ARB's op de niertubuli bij chronisch HF. Tenslotte, hogere dosering en verhoging van de dosering van lisdiuretica tijdens de follow-up duidde op progressie naar eindstadium HF.

Bij patiënten die een harttransplantatie (HTx) ondergaan, blijft postoperatieve acute nierbeschadiging (AKI) een veelvoorkomende complicatie met een slechte prognose (**hoofdstuk 12**). Hoewel nierbeschadiging na harttransplantatie van oudsher wordt toegeschreven aan verminderde arteriële perfusie en nefrotoxiciteit door calcineurineremmers, leveren onze resultaten sterk bewijs voor een onafhankelijke relatie tussen preoperatieve rechtszijdige hemodynamica (lagere pulmonale arteriële pulsatiliteitsindex [PAPi] en hogere rechteratriumdruk [RAP]-waarden) en de ernst van postoperatieve AKI. Deze gegevens suggereren dat preoperatief gecompromitteerde rechtszijdige veneuze druk klinische aandacht verdient in de context van postoperatieve AKI en dat preoperatieve PAPi en RAP-waarden kunnen worden gebruikt als vroege voorspellers van AKI.

In **hoofdstuk 13** gebruiken we onze 23-jaar omvattende registratiegegevens (van 1985 tot 2008) om de korte en lange termijn klinische prognose te onderzoeken van patiënten die zijn opgenomen met acuut HF. Onze resultaten tonen aan dat de langetermijnprognose van patiënten bij wie de nierfunctie behouden blijft, de afgelopen jaren aanzienlijk is verbeterd. De langetermijnprognose bij diegenen met nierfunctiestoornissen is echter verslechterd. We ontdekten ook dat de aanwezigheid van anemie geassocieerd is met een slechte prognose op korte termijn bij alle patiënten. Ten slotte was anemie bij patiënten met renale disfunctie geassocieerd met een verminderde langetermijnprognose.

CONCLUSIES

Dit proefschrift suggereert een veelbelovende rol voor dynamische voorspellingsmodellering met behulp van herhaaldelijk gemeten, klassieke en nieuwe biomarkers bij het inschatten van de prognose van patiënten met chronisch HF.

Eveneens kan statistische interactie-analyse ons helpen om te leren hoe we een interventie het meest effectief kunnen gebruiken, wie wel en wie er niet van zou profiteren, hoe de comorbiditeiten van patiënten het effect beïnvloeden en of het schadelijk zou zijn in specifieke subpopulaties.

Hoewel het falende hart zowel de glomerulaire als ook de tubulaire compartimenten van de nieren aantast, is de mate van beschadiging in deze compartimenten meestal niet simultaan over de tijd. Belangrijk is dat de verslechtering van ofwel het glomerulaire ofwel het tubulaire compartiment, en met name gelijktijdige beschadiging van beide compartimenten, een slechte prognose bij chronisch HF met zich meebrengt. Het patiënt-specifieke beloop van glomerulaire markers (plasma creatinine, eGFR en cystatin C) en tubulaire markers (urinary NAG en KIM-1) voorspelt op dynamische wijze de ongunstige klinische uitkomsten zoals ziekenhuisheropname door HF en overlijden, tijdens de ogenschijnlijk klinisch stabiele fase van HF. In een multi-orgaan syndroom zoals HF kunnen circulerende biomarkers die de multi-orgaan pathofysiologie weerspiegelen een waardevol klinisch hulpmiddel zijn, aangezien deze cellulaire signalen voorafgaan aan decompensatie van het hart en vroege weefsel- en orgaan specifieke informatie kunnen verschaffen. In dit opzicht hebben we verschillende nieuwe biomarkers geïdentificeerd die de potentie hebben om de multi-orgaanpathofysiologie van chronisch HF verder te karakteriseren, maar die ook kunnen helpen bij het monitoren van ziekteprogressie tijdens poliklinische follow-up.

Bij patiënten met ACS wijzen de waarden van plasma cystatin C eerder op verslechtering van de nierfunctie dan creatinine-gebaseerde indicatoren dat doen. Bovendien wijzen hogere cystatin C waarden als prognostische indicator op het opnieuw krijgen van ACS en overlijden, tijdens het eerste jaar na ACS bij patiënten met milde tot matige renale disfunctie. Een dergelijke renale disfunctie modificeert ook de relatie tussen cystatin C en de aanwezigheid van VH-IVUS coronaire laesies met hoog risico. Deze bevindingen mogen niet worden verwaarloosd. Een dergelijke milde nieraandoening vereist meestal geen medische aandacht, maar de subtiele verschillen die door cystatin C worden weergegeven lijken potentie te hebben om de risicostratificatie van patiënten te verbeteren.

Verlagen van de dosis of het niet-geven van ACE-remmers / ARB's uitsluitend op basis van glomerulaire nierfunctiemarkers is niet gerechtvaardigd bij stabiele patiënten met HF vanwege de gunstige cardiale, tubulaire en ontstekingsremmende effecten. Daarentegen, een hogere dosering en verhoging van de dosering van lisdiuretica tijdens follow-up indiceert progressie naar eindstadium HF. Bij patiënten met terminaal HF voorspellen preoperatieve rechtszijdige hemodynamische indicatoren, zoals PAPi en RAP, de ernst van postoperatieve AKI, wat een sleutelrol

suggereert voor chronische renale veneuze congestie bij nierbeschadiging kort na een harttransplantatie.

KLINISCHE PERSPECTIEVEN EN TOEKOMSTIGE AANBEVELINGEN

Dit proefschrift beschrijft aanvullende inzichten in de interacties tussen hart en nieren die mogelijk vroege identificatie en monitoring kunnen verbeteren, om op die manier de risicovoorspelling te verbeteren bij patiënten bij wie deze beide orgaansystemen zijn aangedaan. Dit proefschrift toont met name aan dat biomarkerpatronen in individuen over de tijd extra prognostische informatie bevatten, bovenop de traditionele benadering met een eenmalige (baseline) meting. Voor artsen is het medisch relevant om alle beschikbare informatie (op baseline en tijdens follow-up) te gebruiken om het dynamische ziekteverloop nauwkeurig te detecteren en om individuele prognoses te kunnen stellen. Een dergelijke dynamische prognose zou kunnen worden geïntegreerd in de klinische besluitvorming en zou bijzonder nuttig kunnen zijn voor therapieën die aangrijpen op specifieke weefsels.

Om dit te bereiken rapporteren we de resultaten van een groot aantal klassieke en opkomende biomarkers en behandelen we verschillende fasen van biomarkeronderzoek. Deze fasen omvatten initiële hypothese genererende testen (d.w.z. het beoordelen of specifieke biomarkers significant verschillen tussen patiënten met en zonder ziekte uitkomst), prospectieve validatie (d.w.z. het voorspellen van toekomstige uitkomsten in prospectieve cohortonderzoeken) en beoordeling van de additieve waarde bovenop traditionele risicovoorspellers. Hierdoor biedt dit proefschrift een solide basis voor toekomstige studies om de klinische bruikbaarheid van de hier beschreven biomarkers te onderzoeken, voor een biomarker-geleide behandelingsstrategie. We hopen dat de resultaten van ons werk, zoals beschreven in dit proefschrift, zullen bijdragen aan het verlagen van de mortaliteit- en ziekenhuisopnamecijfers van patiënten, het verbeteren van de kwaliteit van leven bij deze patiënten en ook het verlagen van de kosten voor de gezondheidszorg.

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Pre-graduation period

Petrovic I, Oroz A, **Brankovic M**, Andjelic G. Management of congenital neck malformations – branchial cysts. MD-Medical Data. 2014; 6(2):137-141.

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Summary of PhD training and teaching activities

Name PhD student:	Milos Brankovic
Erasmus MC Department:	Cardiology
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR) Erasmus MC
PhD period:	June 2015 – December 2018
Promotor:	Prof. dr. ir. H. Boersma
Supervisors:	Dr. I. Kardys
	Dr. K. Martijn Akkerhuis

1. PhD training

	Year	Workload (ECTS)
General academic skills		
NIHES Master of Science in Clinical Epidemiology	2015	70.0
Biomedical English Writing and Communication	2017	3.0
Research skills		
NIHES ESP14 Clinical trials	2016	0.7
NIHES ESP15 Topics in Meta-analysis	2016	0.7
NIHES ESP25 Health Economics	2016	0.7
NIHES ESP65 The Practice of Epidemiological Analysis	2016	0.7
In-depth courses		
COEUR Heart Failure Research	2016	1.5
COEUR Arrhythmia Research Methodology	2016	1.5
COEUR Cardiovascular Imaging and diagnostics	2017	0.5
COEUR Intensive Care Research	2017	0.5
Presentations		
American College of Cardiology Annual Session (poster)	2016	0.3
American Heart Association Annual Session (poster)	2016	0.3
European Society of Cardiology Congress (2 x poster)	2017	0.6
American Heart Association Annual Session (poster)	2017	0.3
COEUR symposium - Enhancing precision medicine through protein biomarker profiling (oral presentation)	2017	0.5

International conferences and symposia		
American College of Cardiology Annual Session, Chicago, IL, USA	2016	0.9
American Heart Association Annual Session, New Orleans, LA, USA	2016	1.5
European symposium on ultrasound contrast imaging, Rotterdam, The Netherlands	2017	0.6
European Society of Cardiology Congress, Barcelona, Spain	2017	1.5
American Heart Association Annual Session, Anaheim, CA, USA	2017	1.5
Seminars and workshops		
COEUR & Mivab - Renal cardiac and vascular aging	2016	0.5
COEUR - Right Ventricular Failure	2016	0.2
COEUR PhD day	2016, 2017	0.6
Awards		
Best Master Research Paper Award, NIHES, Erasmus University	2016	
Oral Presentation Winner, COEUR PhD Day, Erasmus University	2016	

2. Teaching activities

	Year	Workload (ECTS)
Lecturing		
Dep. of Nephrology – Renal complications in heart failure	2018	0.1
KLEP - Statistical Interaction analysis	2017	0.1
COEUR PhD day	2016, 2017	1.0
Teaching assistant		
MolMed Basic course SPSS	2016, 2017	3.0
Supervising practical		
Supervising research of PhD candidate	2017	0.4
Others		
Peer review of articles for scientific journals	2016-2018	
Total		93.7

Erasmus MC

Universitair Medisch Centrum Rotterdam

Milos Brankovic, MD, has completed his PhD thesis on the heart-kidney interactions in acquired heart disease.

Born in Belgrade, Serbia, he studied medicine at the School of Medicine, University of Belgrade, where he was elected Editor-in-Chief of the student scientific journal "Medical Youth" and Vice-President of Organizing Committee of the first Global Students Conference of Biomedical Sciences in Belgrade. Having achieved a ranking in the top 1% of his class, he received a two-month scholarship in 2013 to train under the mentorship of Dr. Bud Frazier at the Texas Heart Institute, in Houston. In 2014, he received a four-month research scholarship at the Netherlands Institute for Health Sciences (NIHES), Erasmus MC, Rotterdam. For his work, he was awarded several times including the "Nikola Spasic" award for the best-graduated medical student at the University of Belgrade. In 2014, he obtained his MD degree with an average grade of 10,00/10,00. Subsequently, he completed his internship at the Clinic for Vascular and Endovascular Surgery, Clinical Center of Serbia, in Belgrade, where he has authored four peer-reviewed publications on different vascular pathologies. In June 2015, he started working on his PhD thesis at the Department of Cardiology, Erasmus MC, under the mentorship of Prof. Eric Boersma. In February 2016, he studied at the Institute of Public Health, University of Cambridge, in Cambridge, as a part of the NIHES MSc program. In September 2016, he obtained his MSc degree in Clinical Epidemiology at the NIHES, where he was awarded for the best master's research paper.

His research interests include coronary artery disease, heart failure, heart transplantation, mechanical circulatory support, chronic kidney disease, aortic diseases, and vascular malformations with a focus on clinical-decision making, biostatistics, and epidemiology.