Aus der Medizinischen Klinik mit Schwerpunkt Kardiologie der
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DISSERTATION

Metabolomic biomarkers for risk prediction in chronic heart failure

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1. Abstract

1.1 Abstract (Deutsch)

Einleitung: Chronische Herzinsuffizienz (CHF) ist weltweit eine der Hauptursachen für Morbidität und Mortalität, und ihre Prävalenz steigt in Industrieländern weiter an. Ein wichtiger Aspekt des CHF-Managements besteht darin, sicherzustellen, dass Ärzte und Patienten über die erforderlichen Ressourcen verfügen, um die besten Gesundheitsentscheidungen zu treffen. Prognostische Biomarker sind eine solche Ressource. Das n-terminale natriuretische Peptide (NT-proBNP) ist als Goldstandard-Biomarker für die Prognose anerkannt. Einige Studien haben jedoch eine hohe intraindividuelle Varianz und hohe Referenzänderungswerte bei CHF-Patienten berichtet. Metabolite Profiling oder Metabolomics können dazu beitragen, den Bedarf an robusteren prognostischen Biomarkern zu decken. Das Cardiac Lipid Panel (CLP) ist ein neu entdecktes Panel von Biomarkern auf Metabolitenbasis, von denen zuvor gezeigt wurde, dass sie den diagnostischen Wert von NT-proBNP verbessern. Über den prognostischen Wert ist jedoch wenig bekannt.

Ziele: Wir haben versucht, die folgenden Fragen zu metabolomischen Biomarkern in CHF zu beantworten:

• Welche metabolomischen Biomarker haben zuvor gezeigt, dass sie die Prognose von CHF-Patienten verbessern, und um wie viel?
• Fügt das neuartige CLP einen prognostischen Wert für die Vorhersage der 4-Jahres-kardiovaskulären Mortalität hinzu?
• Wie verhält sich der CLP-Risiko-Score im Vergleich zu anderen etablierten kardiovaskulären prognostischen Scores?

Methoden: Die durchgeführten Analysen waren systematische Überprüfung und Metaanalyse, gefolgt von zwei Teilstudien zur Ergebnisvorhersage der CIBIS-ELD-Studie. Die CIBIS-ELD war eine doppelblinde, multizentrische Studie bei älteren CHF-Patienten, die randomisiert auf Bisoprolol und Carvedilol umgestellt wurde. Eine gezielte metabolomische Analyse der drei CLP-Biomarker wurde an Basisserumproben (n = 280) durchgeführt und ihr prognostischer Wert wurde nach 4 und 10 Jahren Follow-up bewertet.

Ergebnisse: Aus 19 Studien (45.420 Probanden, 5.954 Ereignisse) waren insgesamt 39 von 41 Metaboliten mit einer kombinierten Effektgröße von 1,14 (1,07-1,20) signifikant. Die durchschnittliche Änderung der c-Statistik nach Zugabe der Biomarker betrug 0,0417 (SE 0,008). In der 4-Jahres-Follow-up-Studie erreichten 35 (18%) Probanden den primären Endpunkt des kardiovaskulären Todes. Die AUC für das Modell mit nur NT-proBNP betrug 0,86 und das Modell mit CLP plus NT-proBNP betrug 0,90. In der 10-Jahres-Follow-up-Studie erreichten 95 (34%) Probanden den primären Endpunkt. Die IAUC für FRS betrug 0,53, SHFM 0,61, MAGGIC 0,68, BCN Bio-HF 0,72 und CLP 0,78.

Schlussfolgerung: Bei Patienten mit CHF verbesserte die Einbeziehung eines Panels von 3 Biomarkern auf Metabolitenbasis in einen Risiko-Score den prognostischen Nutzen von NT-proBNP und übertraf andere kardiovaskuläre Risiko-Scores. Dieser neuartige Ansatz verspricht eine Verbesserung der klinischen Risikobewertung bei CHF-Patienten.

1.2 Abstract (English)

Background: Chronic heart failure (CHF) is a leading cause of morbidity and mortality worldwide and its prevalence continues to rise in developed countries. An important aspect of CHF management is to ensure that clinicians and patients have the necessary resources to make the best health decisions. Prognostic biomarkers are one such resource. N-terminal pro–B type natriuretic peptide (NT-proBNP) is recognized as the gold standard biomarker for prognosis, but some studies have reported a high intra-individual variance and high reference change values among CHF patients. Metabolite profiling, or metabolomics, can help meet the need for more robust prognostic biomarkers. The Cardiac Lipid Panel (CLP) is a newly discovered panel of metabolite-based biomarkers that has previously shown to improve the diagnostic value of NT-proBNP. However, little is known about its prognostic value.

Aim: We sought to answer the following questions concerning metabolomic biomarkers in CHF:

- Which metabolomic biomarkers have previously shown to improve prognosis of CHF patients, and by how much?
- Does the novel CLP add prognostic value for prediction of 4-year cardiovascular mortality?

- How does the CLP risk score perform in comparison to other established cardiovascular prognostic scores?

Methods: The performed analyses were systematic review and meta-analysis followed by two outcome prediction sub-studies of the CIBIS-ELD trial. The CIBIS-ELD was a double blind, multicenter trial in elderly CHF patients, randomized to bisoprolol and carvedilol. A targeted metabolomic analysis of the three CLP biomarkers was performed on baseline serum samples (n=280) and its prognostic value was evaluated at 4-year and 10-year follow up.

Results: From 19 studies (45,420 subjects, 5,954 events) a total of 39 of 41 metabolites were significant with a combined effect size of 1.14 (1.07-1.20). The average change in c-statistic after adding the biomarkers was 0.0417 (SE 0.008). For the 4-year follow up study, 35 (18%) subjects met the primary endpoint of cardiovascular death. The AUC for the model with NT-proBNP only was 0.86, and the model with the CLP plus NT-proBNP was 0.90. For the 10-year follow up study, 95 (34%) subjects met the primary endpoint. The IAUC for FRS was 0.53, SHFM 0.61, MAGGIC 0.68, BCN Bio-HF 0.72, and CLP 0.78.

Conclusion: In patients with CHF, incorporating a panel of 3 metabolite-based biomarkers into a risk score improved the prognostic utility of NT-proBNP and outperformed other cardiovascular risk scores. This novel approach holds promise to improve clinical risk assessment in CHF patients.

2. Background

Chronic heart failure (CHF) is a leading cause of morbidity and mortality and its prevalence continues to rise, partly because of a shift in the age distribution and improved care and treatment (1, 2). Clinicians should ensure that patients with CHF have the necessary resources and knowledge to make the best health decisions. Resources which support clinical decision-making, such as prognostic biomarkers, could help in making such shared decisions. Amongst biomarkers widely used in CHF, N-terminal pro–B type natriuretic peptide (NT-proBNP) is recognized as a standard reference for both prognosis and diagnosis. Despite the widespread use of NT-proBNP, some studies have reported a high intra-individual variance among patients with CHF, along with high reference change values for this biomarker (3-5). Recently, there has been an increase in the number of prognostic biomarkers being tested for CHF such as growth differentiation factor-15 (GDF-15) (6, 7), high-sensitivity C-reactive protein (hs-CRP) (8, 9), galectin-3 (Gal-3) (10, 11), and high-sensitivity troponin T (hs-TnT) (12, 13). However, long-term follow-up studies are lacking, and the added value of these markers is still under debate.

Metabolomic profiling, or metabolomics, can help meet the need for more precise prognostic biomarkers. Metabolomics is the study of small-molecule metabolites (<1,500 Da) that provides a snapshot of a deeper phenotype that reflects the products of cellular metabolism and the effects of genomic, transcriptomic, and proteomic variations. This approach provides a holistic signature of biochemical activities that could be associated with diet, medication, and disease progression (14-16). The advantages of metabolomics over the other omics technologies include its high sensitivity and the smaller size of the number of endogenous molecules relative to the number of genes, mRNA, or proteins. One of the disadvantages of metabolomics technology, is quantification. The signal intensity of any compound is affected by the type of sample preparation and its molecular environment, therefore, results across experiments may be heterogenous. Nevertheless, studies of metabolomic biomarkers in CHF have been published previously that support the overall hypothesis that circulating metabolites may be used for the diagnosis and prognosis of cardiovascular disease patients (17-28).

Metabolomic biomarkers can be incorporated into a prognostic model, defined

as a formal combination of multiple predictors from which risk of a specific outcome can be calculated for individuals. Prognostic models are abundant in the literature, and the most popular ones include the SHFM (Seattle Heart Failure Model), FRS (Framingham Risk Score), MAGGIC (Meta-analysis Global Group in Chronic Heart Failure) and BCN Bio-HF (Barcelona Bio-Heart Failure Risk Calculator). The SHFM score is the most thoroughly validated and contains the most predictor variables of the four (29). The MAGGIC score (30) was developed from a dataset of over 39,000 patients across 30 studies and validated on more than 60,000 patients from 2 CHF cohorts (31, 32). The FRS score was developed as a sex-specific risk score that can be conveniently used to calculate incident cardiovascular disease (CVD) risk or risk of individual CVD events (33). The BCN Bio-HF score contains 11 clinical variables which includes the most biomarker variables (NT-proBNP, high-sensitivity cardiac troponin T (hs-cTnT), high-sensitivity soluble ST2 (ST2)) and has been validated (34, 35). These models all use common clinical variables for the prognosis of CHF patients and have convenient online calculators. Although these scores have been validated, they have not been widely adopted possibly because they are not routinely calculated in clinical practice (36-38), have poor reliability at the individual patient level (31), or suffer from a significant amount of missing data requiring imputation. Developing a score utilizing metabolomic biomarkers in combination with conventional cardiovascular biomarkers may improve result in an improved prognostic model.

Recently, a novel panel of metabolites known as the Cardiac Lipid Panel (CLP) was found to improve the diagnostic performance over NT-proBNP alone (39). Details of the CLP have been published previously (39). Briefly, the CLP is a biomarker panel consisting of three specific metabolomics features: triacylglycerol (TAG) 18:1/18:0/18:0, phosphatidylcholine (PC) 16:0/18:2, and the sum of the 3 isobaric sphingomyelin (SM) species SM d18:1/23:1, SM d18:2/23:0, and SM d17:1/24:1. Its prognostic performance, however, is unknown. In this study, the predictive value of the CLP was analyzed in elderly ambulatory patients with CHF at 4 year follow up and 10 year follow up.

3. Aims

The presented work aimed to answer the following three questions:

1. Which metabolomic biomarkers have previously shown to improve prognosis of CHF patients, and by how much?
2. Does the novel CLP add prognostic value for prediction of 4-year cardiovascular mortality?
3. How does the CLP risk score perform in comparison to other established cardiovascular prognostic scores?

4. Methods

Data used in this work was obtained from previously published metabolomics studies of prognostic biomarkers, for Aim 1; and the CIBIS-ELD (The Cardiac Insufficiency Bisoprolol Study in ELDerly) trial for Aims 2-3. CIBIS-ELD was an investigator-initiated, multicenter trial (55 centers in four countries), 1:1 randomized, double-blind phase 3 trial in CHF patients, which compared the tolerance of bisoprolol and carvedilol (40). Patients were 65 years or older, and beta-blocker naïve or on 1/4 of the guideline recommended target or equivalent dose at baseline. In total, 883 patients were enrolled and randomized to bisoprolol (10 mg/day target dose) or carvedilol (25 mg b.i.d target dose) (40). For aims 2-3 a random sub-cohort (n=589) was selected. Then selection for only those which passed sample quality control (41), and had a sufficient amount of blood aliquot sample for metabolite profiling, resulting in a final cohort of 280 cases. The final cohort was studied in a case-cohort design. The primary outcome, cardiovascular death, was defined as death by myocardial infarction, non-responding arrhythmia, asystole, chronic pump failure, or other cardiac cause and verified by a blinded committee of cardiologists.

Aim 1: Systematic Review and Meta-analysis

To answer Aim 1, a systematic review and meta-analysis of metabolomic biomarkers for CVD risk prediction was performed. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline was followed for the systematic review and meta-analysis. Multiple layers of keyword search criteria were used in the PubMed, Google Scholar, SCOPUS, and Web of Science databases from January 2010 to July 2019. The inclusion and exclusion criteria were as follows:

Inclusion criteria:

- Human blood samples
- Prognosis of CVD outcomes
- Multivariate prediction models
- Discrimination or risk reclassification analysis of prediction models
- Mass spectrometry (MS) or Nuclear Magnetic Resonance (NMR) approaches

Exclusion Criteria:

- Diagnostic biomarker studies
- Literature reviews
- Non-CVD Studies
- Articles published in languages other than English

Aim 2: CIBIS-ELD 4-year Follow up Study

To answer Aim 2, data from the CIBIS-ELD sub-cohort (n=280) at 4-year follow up was used and the CLP biomarker panel and NT-proBNP was measured. A simple score ranging from 0-4 was calculated using the three CLP biomarkers plus NT-proBNP. Time-to-event analysis and discrimination analysis of 3 multivariate models were performed:

- Model A: Clinical Variables
- Model B: Clinical Variables + NT-proBNP
- Model C: Clinical Variables + NT-proBNP + CLP

Risk reclassification was measured using the Net Reclassification Index (NRI) and Integrated Discrimination Index (IDI).

Aim 3: CIBIS-ELD 10-year Follow up Study

To answer Aim 3, the same subjects from the CIBIS-ELD study were used but at 10-year follow-up. The CLP score prognostic performance was compared to four traditional prognostic scores: FRS, MAGGIC, BCN-BioHF, and SHFM. Each prognostic score was calculated using the corresponding method proposed by the original authors (29-31, 34). The CLP risk score was calculated the same way as the 4-year follow up study and compared to the other scores using, time-to-event analysis, differences in

discrimination statistics, and cluster analysis.

Biomarker Measurements

Baseline blood samples were taken in standardized conditions by venous puncture after a 20-minute resting period, centrifuged immediately and stored at -80 degrees Celsius. The samples were transferred on dry ice to a specialized metabolomics lab for analysis. Targeted metabolite profiling was performed using a commercially available kit. The kit uses a protocol based on a 1-phase extraction of the blood samples followed by gas chromatography mass spectrometry (GC-MS) (Agilent 6890 GC coupled to an Agilent 5973 MS-System) and liquid chromatography tandem-mass spectrometry (LC-MS/MS) (Agilent 1100 HPLC-System coupled to an Applied Biosystems API4000 MS/MS-System) analysis as previously described (39). The analytical protocol was designed for routine measurement in the clinical practice setting; however, it is currently only available in specialized labs equipped with MS technology. NT-proBNP was also measured from the same blood samples using commercially available assays (Elecsys, Roche Diagnostics).

Statistical Analysis

Aim 1: Systematic Review and Meta-analysis

For the meta-analysis, the random-effects model was used because it incorporates both within- and between-study components of variance. Heterogeneity for effect sizes was estimated by the Cochran Q test and I2 statistic, with .30% considered at least moderate heterogeneity. Publication bias was assessed by visual inspection of funnel plots and regression test for funnel asymmetry, evaluating skewness of the distribution of SEs around the effect estimates, using a significance level of $P < 0.05$ to indicate significant asymmetry. Meta-analysis was conducted using metan package for Stata 14.2 (StataCorp) with a two-tailed of 0.05 considered statistically significant. Publication bias and heterogeneity was conducted using the R 3.5.0 (R Core Team, 2018) metafor package (v2.13.1; Gabry & Goodrich, 2016).

Aim 2: CIBIS-ELD 4-year Follow up Study

The CLP risk score was calculated as the sum of biomarkers above the Youden index cut-off (42). There were 4 cut-off values, since four biomarkers are included in the score, three from the CLP and one from NT-proBNP. Each cut-off was calculated using Youden's index of the predicted probability from the Cox regression. To evaluate the predictive value of the CLP score, three multivariable prediction models were built using Cox regression. The first model was built using only clinical covariates (Model A); then NT-proBNP was added to the first model (Model B); finally, the CLP score was added to Model B (Model C). The considered clinical covariates were: age, sex, BMI, New York Heart Association (NYHA) class, creatinine, LDL cholesterol, triglycerides, left ventricular ejection fraction (LVEF), history of diabetes, history of myocardial infarction, smoking history, hypertension, hyperlipidemia, coronary artery disease, medication including beta-blockers, aldosterone receptor blockers, ace inhibitors, anti-arrhythmic agents, aspirin, calcium channel blockers, diuretics, glycosides, nitrates, statins, sedative agents, vitamin K antagonists. To measure the discrimination of each model, the AUC was calculated for Models A, B, and C. Differences in Uno's concordance statistics were calculated for hypothesis testing of the change in AUC of the three models (43).

To measure risk re-classification, both continuous and categorical NRI were calculated as well as IDI (44, 45). The categorical NRI used 3 risk categories of <60%, 60%-85% and >85% corresponding to low, intermediate, and high risk. The continuous NRI does not depend on the choice of categories but allocates any change in predicted risk in the correct direction (46). IDI measures the ability of the new model to increase average sensitivity without reducing average specificity.

Aim 3: CIBIS-ELD 10-year Follow up Study

The same sub-cohort was analyzed at 10-year follow up. Cox Regression was performed on each of the prognostic scores to measure their association with the outcome. Harrell's c statistic (47) and IAUC were generated to assess the discrimination of each score. The IAUC curves are computed as a weighted average of the AUC values at all the event times, with the weights as the jumps of the Kaplan-Meier estimate of the survivor function. Hypothesis testing of the change in IAUC was performed by calculating the differences in concordance statistics (43). . Calibration (i.e., the agreement between observed outcomes and predictions) of all models was assessed

graphically, with calibration plots. Analyses were performed using SAS statistical software version 9.4 (SAS Institute, Inc., Cary, North Carolina) and JMP pro software version 14, R software version 3.6.1, and Stata Statistical Software version 16 (48-51).

Hierarchical cluster analysis was performed using the 5 prognostic scores (CLP, FRS, MAGGIC, BCN-BioHF, SHFM) as input to assess whether the risk scores could be used partition subjects into different risk groups. Using this clustering technique, similar prognostic score data from participants were grouped together, such that the members in the same group were more similar to each other than the members in the other groups. Comparisons of patient characteristics across risk clusters was performed using Wilcoxon rank sum test for continuous variables; and Pearson's chi-square test (or Fisher's exact test) or Mantel-Haenszel Chi-square test for categorical and ordinal data, respectively. Kaplan-Meier curves were used to compare the survival distribution across risk clusters.

5. Results

Aim 1: Systematic Review and Meta-analysis

The combination of search filters based on the inclusion/exclusion criteria retrieved 604 articles from Pubmed, 52,700 from Google Scholar, 44,591 from SCOPUS, and 663 from Web of Science. Among those there were 1,333 unique abstracts reviewed and 1,311 were excluded. After final exclusions, 22 publications were eligible for further analysis. The mean age per study ranged from 45 to 85 years. The mean follow-up time ranged from 1 to 23 years and the number of participants ranged from 67 to 8,101. The majority of studies used a cohort design in at least 1 of their analyses, with only 2 exclusively using a case-control design (52, 53). Most studies used a targeted metabolite profiling approach and only 4 studies used an untargeted approach (19, 24, 52, 54). Most studies used MS technology with only 2 studies which used exclusively NMR technology (24, 55), and 1 study which used both and then measured the consistency between the two methods for biomarker associations with CVD outcomes (28).

The meta-analysis included the effect sizes of 41 biomarkers from 18 studies

(45,420 subjects, 5,954 events). **Figure 1** shows the forest plot of effect sizes and 95% CI of the selected metabolomic features and metabolite classes (subgroup analysis) associated with fatal CVD outcomes. The combined effect for all the metabolites was 1.14 (1.07-1.20), but the heterogeneity was high (I2 = 91.5% p < 0.0001). A total of 39 biomarkers were significantly associated with fatal CVD outcomes, of which 27 were associated with a higher risk and 12 were associated with a lower risk. The median number of metabolites used in each study's prediction model was 4 and most initially analyzed between 30 and 300 metabolites, except for 3 studies which focused on one metabolite, TMAO, (17, 52, 56). The definition of CVD outcomes varied between studies; 15 studies used a composite outcome, and most studies had a mortality component (n=20). The c-statistics/AUC for multivariate models including metabolomic biomarkers ranged from 0.684 to 0.874, and the average change in c-statistics after adding the metabolite biomarkers was 0.0417 (SE 0.008). The only subgroups with significant combined effect sizes were acylcarnitines ES 1.15 (1.08-1.23), I2 = 23.4% (p=0.265), and metabolite scores ES = 2.09 (1.49-2.93), I2 = 79.2% (p=0.001). Publication bias was significant as the regression test indicated funnel plot asymmetry (p = 0.0020).

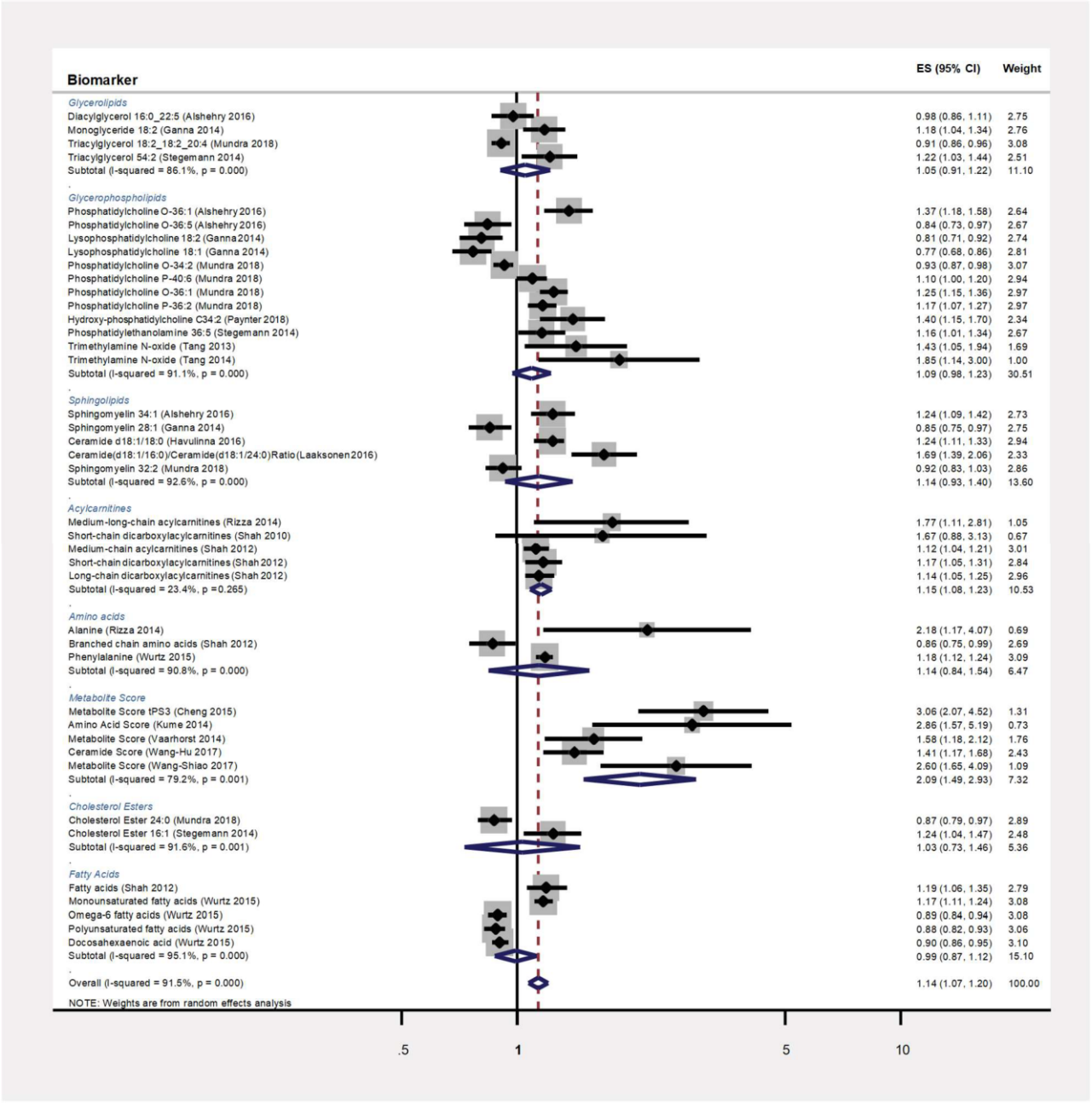

Figure 1: Forest plot of effect sizes of metabolomic biomarkers and combined effect sizes for subgroups in predicting CVD outcomes (McGranaghan *et al.*, 2020)

Aim 2: CIBIS-ELD 4-year Follow up Study

Aim 2: Does the novel CLP add prognostic value for prediction of 4-year cardiovascular mortality?

Mean patient age was 72.1 (4.9) years, 73.6% were men, 45% patients had heart failure with reduced ejection fraction (HFrEF) (LVEF < 35%), 49% had heart failure with mid-range ejection fraction (HFmEF) (LVEF 35-49%), 4% had heart failure with preserved ejection fraction (HFpEF) (LVEF ≥ 50%), and the majority of patients were in

NYHA functional class II (67.5%). During the follow-up period (mean = 50 months, SD = 8; median = 46 months), 35 (13%) died from cardiovascular causes.

Figure 2 shows the measures of discrimination (AUC) for the 3 multivariable models with a comparison to its preceding model to test the level of significance of the change in AUC after adding the respective covariate(s). The AUC for Model A was 0.84 and that of Model B was 0.86 and the final adjusted Model C was 0.90. The difference in AUC after adding the CLP score (Model B vs. C) was significant (p = 0.02), whereas the difference after adding NT-proBNP to the clinical model (Model A vs. B) was insignificant (p = 0.47).

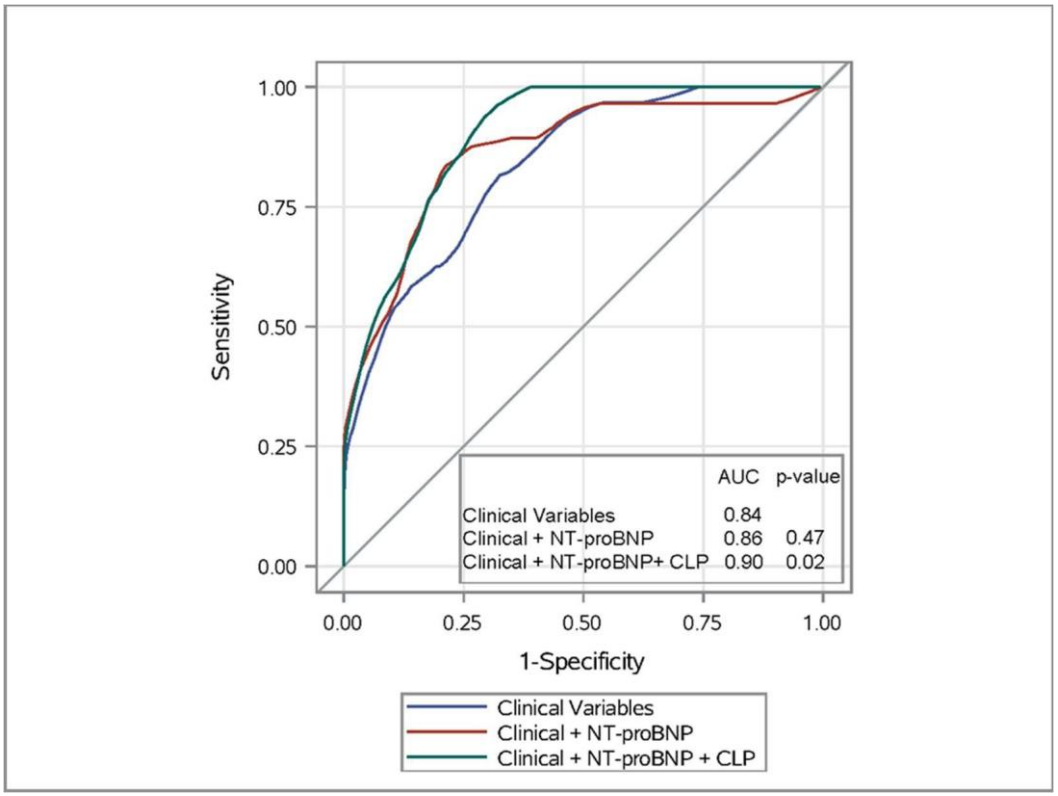

Figure 2: Discrimination analysis of the CLP biomarker risk score for 4-year cardiovascular mortality (McGranaghan *et al.*, 2020)

The overall categorical NRI was 0.25 using the 3 risk categories 0-60%, 60-85% and >85%, meaning 25% of subjects were re-classified into the respective correct risk category after adding the CLP. Accordingly, 59% of the re-classified cases were down-graded, and the other 41% were up-graded. Amongst patients experiencing events, the overall categorical NRI was 0.60, with 33% of those down-graded and 67% up-graded. For non-events, the categorical NRI was 0.19, with 70% of those down-graded and 30% up-graded. The overall continuous NRI was 0.472 and the IDI was 0.019. The CLP model (Model C) showed that its high-risk category contained predominantly subjects who experienced an event (77%), whereas the respective fraction in the NT-proBNP

model (Model B) was only 42%.

Aim 3: CIBIS-ELD 10-year Follow up Study

Aim 3: How does the CLP risk score perform in comparison to other established prognostic scores at 10 year follow up?

During the follow-up period (mean=81 months, SD=33; median=96 months), 95 (34%) patients met the primary outcome. **Table 1** shows the Cox Regression results. The CLP (HR =2.38, p<0.001), SHFM (HR = 2.01, p=0.002, and MAGGIC (HR =1.10, p<0.001), and BCN Bio-HF (HR=1.09, P=0.0393) scores were significantly associated with the outcome while FRS was not.

Score	**HR (95% CI)**	**p value**
SHFM	1.89 (1.29-2.807)	0.0017
FRS	1.02 (0.97-1.07)	0.5291
MAGGIC	1.10 (1.05-1.14)	<.0001
BCN Bio-HF	1.09 (1.00-1.84)	0.0393
CLP	2.38 (1.95-2.92)	<.0001

Table 1: Prognostic Scores and Hazard Ratios for Cardiovascular Mortality

Figure 3 shows the AUC change over time (IAUC) for the 5 prognostic scores with the comparison of concordance statistics for hypothesis testing. The IAUC was 0.53, 0.61, 0.68, 0.72, and 0.78 for FRS, SHFM, MAGGIC, BCN Bio-HF, and CLP, respectively. The models showed adequate calibration except for FRS (calibration curve slope = 0.894).

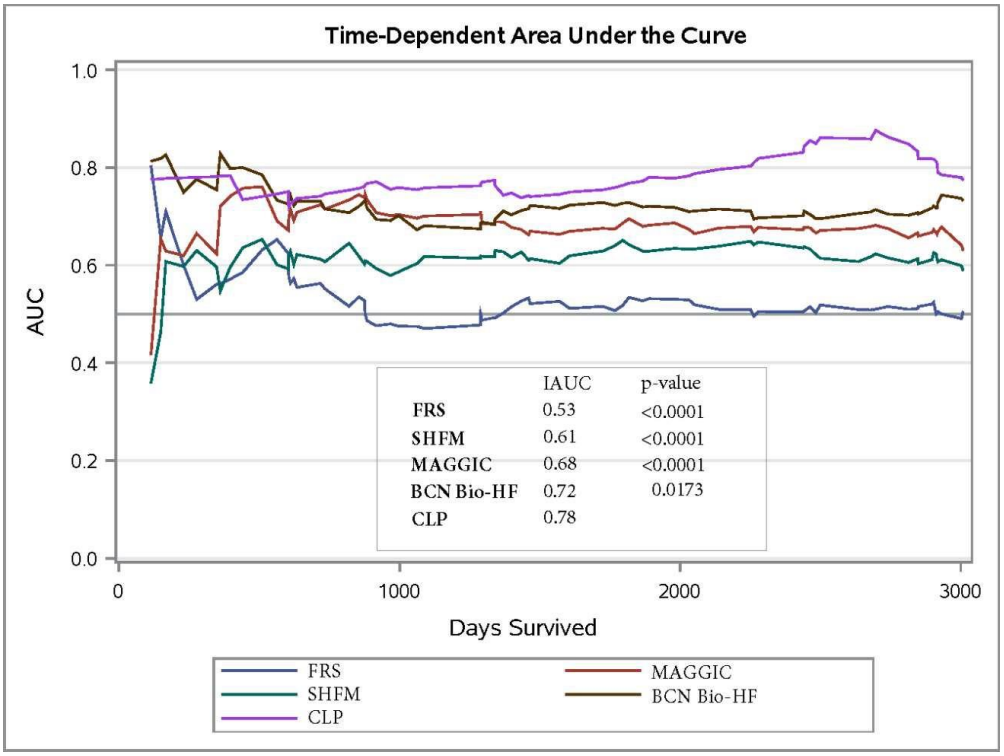

Figure 3: Discrimination Performance for each Prognostic Score for 10-year Cardiovascular Mortality (McGranaghan *et al.*, 2021)

Three risk clusters were generated following hierarchal cluster analysis: low risk, n=119; moderate risk, n= 44; high risk, n=117. Patients in the highest risk cluster were older, with lower LVEF, higher NT-proBNP, and experienced a higher frequency of events. Rates of mortality were: low risk cluster (20%), moderate risk cluster (27%) and high-risk cluster (50%). All prognostic scores' distributions were significantly different across their respective risk clusters. Of the traditional risk scores, only SHFM and MAGGIC, had its highest mean score in the high-risk cluster. The categorical CLP score showed a skewed distribution of higher risk scores (3-4) in the moderate and high-risk clusters. In the high-risk cluster, most subjects had CLP scores of 3-4. **Figure 3** shows the hierarchical cluster dendrogram mapped to illustrate the assignment of patients into their respective clusters with the associated color map which shows the distribution of each prognostic score within each cluster. We can infer from the cluster memberships that the CLP risk score was better at dividing patients into their respective risk cluster.

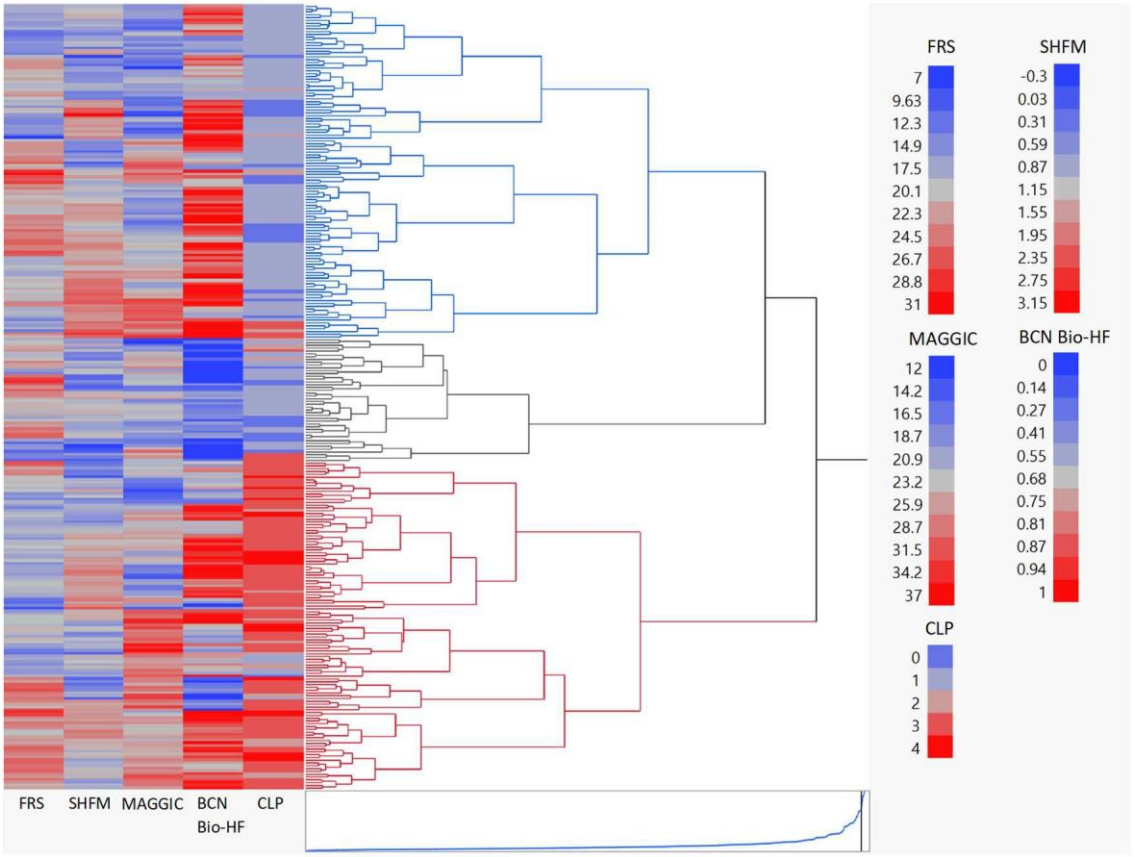

Figure 3: Hierarchal cluster dendrogram of three risk clusters illustrating the assignment of patients into risk clusters based on the prognostic scores. Blue dendrogram indicates the cluster 1 (low risk), n=119; Grey dendrogram indicates cluster 2 (moderate risk), n= 44, Red dendrogram indicates cluster 3 (high risk), n= 117 (McGranaghan *et al.*, 2021)

6. Discussion

Recently the research of biomarkers has increased significantly since it has consistently been shown that a combination of multiple molecules may be more precise in targeting high risk patients. Research groups have reported on metabolomics based clinical investigations to identify prognostic biomarkers, however there is a lack of a centralized metabolite biomarker repository that can be used for meta-analysis and biomarker validation. The aim of the meta-analysis was not to identify, nor to verify new metabolomic prognostic markers, but to report metabolomic biomarkers that are commonly associated with CVD events and assess their added predictive value to provide a basis for comparison of a new biomarker panel (e.g. CLP) studied in Aims 2-3. We found that lipid-based metabolic pathways are the primary focus in cardiovascular metabolomic research and thus represent a valuable target potentially amenable to

clinical use (57-59). The majority of studies measured metabolites using MS technology, which enables higher resolution phenotyping of human blood samples compared to other technologies such as NMR and is also the preferred method for analysing lipid-related metabolic pathways. Metabolite scores were the best performing subgroup, but this should be interpreted with caution since they showed high heterogeneity and were developed in an unstandardized manner specific to the study's sample. Data pre-processing methods, including scaling and transformations, could be another source of heterogeneity. The variety in statistical approaches show the need for clearer analytical standards that should be applied in metabolite-based biomarker score development.

In the second part of the analysis, we showed that a metabolite-based score based on a novel panel of lipid metabolites, known as the CLP, added prognostic value for the prediction of long-term cardiovascular mortality over NT-proBNP alone. Using a biomarker score rather than including the individual CLP biomarkers (n=3) in the model was based on the meta-analysis findings in which the metabolic score subgroup performed better compared to individual biomarkers. The predictive model results for the CLP score were consistent with the meta-analysis as far as incremental prognostic value added (ΔAUC). Adding the CLP to the NT-proBNP model yielded a significant change in AUC and risk reclassification was improved as it correctly identified a higher proportion of high-risk patients experiencing an event. Since NT-proBNP is a marker of elevated left atrial pressures and volume overload, it may be of limited use in well compensated, clinically stable heart failure patients. Application of a single biomarker such as NT-proBNP for outcome prediction is primarily limited by insufficient specificity, resulting in a high false positive rate or low positive predictive value (60, 61). A combination of several metabolomic biomarkers into a panel or a score, may provide a better prognosis over single biomarkers. Therefore, this marker may provide additional prognostic value and more information at the metabolic level.

In the third part of the analysis, we compared the CLP risk score to other conventional CVD risk scores, as we may have missed specific combinations of predictor variables which are used in these scores. The CLP score showed the best discrimination compared to the other 4 scores in predicting 10-year cardiovascular mortality. This indicates that the biomarker information included in the CLP score could more precisely classify high risk CHF patients than the information included in the 4 other risk scores. However, the biomarker information from the CLP is not as easily

obtainable and these findings should first be validated in larger cohorts. In addition, none of the other scores were originally developed for 10-year cardiovascular mortality, but we are not aware of a score specific for predicting 10-year risk of cardiovascular death. Nevertheless, the other risk scores could be improved by incorporating biomarkers. For instance, NT-proBNP is a well-established biomarker and only BCN Bio-HF contained this marker. BCN Bio-HF was the next best performing prognostic score after the CLP. Following discrimination analysis, we performed cluster analysis to assess how well the risk scores could partition subjects into different risk groups. The CLP score showed a more uniform grouping of patients according to their risk score stratification while the other scores showed a more heterogenous distribution across risk clusters. Several prior studies have used similar clustering methods to identify clinically relevant patient subgroups for CHF (62, 63). This approach is useful in defining relevant groups of patients and could minimize the problems of multicollinearity while evaluating whether the predictive variables are useful in separating these groups.

In addition to investigating the improvement of the prognostic performance of cardiovascular outcomes, it is conceivable that metabolomics findings may also foster a better understanding of the pathophysiology and biological mechanisms involved in the development of CHF events. These studies' findings are consistent with previous research of such mechanisms. The CLP metabolites belong to three different lipid subclasses, sphingomyelin (SM) phosphatidylcholine (PC), and triglycerides (TAG), which have been found to be associated with cardiomyocyte stress/apoptosis (64), intestinal microbial metabolism/inflammation (17), and coronary artery disease (65), respectively.

Sphingomyelins and their hydrolysis by sphingomyelinase lead to increased amounts of ceramide. Dysfunctional sphingomyelin and ceramide metabolism may lead to or aggravate cardiovascular diseases via the generation of reactive oxygen species (ROS) involved in the modulation of cell proliferation and apoptosis, neutrophil adhesion to the vessel wall, and vascular tone (66). It was previously reported that lipid species such as Cer-16 and SM-16 were associated with increased risk of heart failure (67). Sigruener et al. reported that the sphingomyelin species SM 16:0, 16:1, 24:1 and 24:2 were associated with mortality [57]. The CLP biomarker panel consists of the sum of three monosaturated fatty acid carrying SM species: SM d18:1/23:1, SM d18:2/23:0, SM d17:1/24:1.

PC is the most abundant lipid in humans and is subjected to lipid peroxidation and ROS formation (68). Myocardium suffers heavily from lipid peroxidation related injury (69). PC carrying polyunsaturated fatty acids such as PC (16:0/18:2), which is a component of the CLP panel, are at high risk for lipid peroxidation (70). Previous studies have shown that lipid peroxidation and ROS generation are associated with cardiac damage and increased risk of mortality. For example, it was found that higher consumption of PC increased the risk of organ injury and cardiovascular mortality (71). Natural antioxidants like α-tocopherol have shown to reduce such oxidative stress and inflammation thereby preventing the progression of cardiac injury (72).

The molecules of TG regulate insulin-signaling pathways by activating serine/threonine kinases, which suppress insulin receptors, and thus inducing peripheral insulin resistance. It has previously been shown that insulin resistance leads to inflammation and atherosclerosis (73). The relationship between total triglycerides and insulin resistance and CVD risk are well established (74), however, the relationships between individual serum TGs and insulin resistance is not. A previous study on individual TGs revealed that serum TG molecules containing saturated and monounsaturated fatty acids, such as TG(16:0/16:0/18:1) and TG(16:0/18:1/18:0), were correlated with insulin resistance. The CLP consists of the saturated and monosaturated fatty acid carrying TAG 18:1/18:0/18:0. These findings indicate that metabolomic studies may help gain a deeper understanding of the molecular mechanisms of CVD.

In future studies we would like to further elucidate the prognostic utility of the CLP and validate its clinical effectiveness by including a larger cohort with more women, patients with early-stage CHF. Following these studies and regulatory approval, it is possible that the CLP can be tested alongside NT-proBNP in the clinical setting for a more precise risk assessment of CHF patients (Figure 5). More accurate risk assessment and prognostic biomarkers in clinical practice could help to match the intensity or type of therapy with an individual patient’s risk. The CHF patient visiting their cardiologist is ordered the CLP test along with the standard NT-proBNP test. The tests’ output would classify the patient’s risk more precisely, supporting the physician’s decision on how to adjust medication therapy or have a more informed discussion with the patient. Currently, the CLP test must be performed at a specialized lab with MS equipment since it is pending formal utilization review.

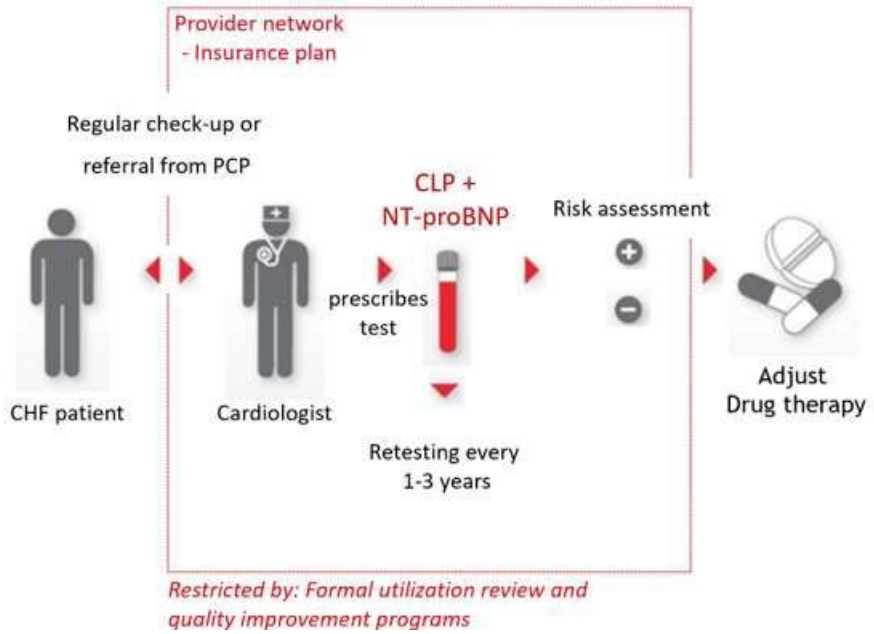

Figure 5: Proposed patient flow for testing the CLP in addition to NT-pro-BNP (McGranaghan *et al.*, 2020)

7. Conclusions

Our findings demonstrate that the CLP risk score comprising a panel of 3 lipid-based metabolomic features meaningfully improved the prediction of CV mortality and outperformed traditional prognostic scores. Results for the improvement in prognostic value were consistent with previous similar studies as shown in the systematic review and meta-analysis. This the metabolomics approach may potentially translate into clinical applications such as routinely applied risk stratification and targeted treatments for CHF patients. The CLP score is a step in the direction of providing a more precise decision support tool to assist clinicians and patients in managing their CHF treatment.

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9. Statutory Declaration

“I, Peter McGranaghan, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic Metabolomic biomarkers for risk prediction in chronic heart failure (Metabolomische Biomarker zur Risikoprognose bei chronischer Herzinsuffizienz), independently and without the support of third parties, and that I used no other sources and aids than those stated. All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

10. Declaration of your own contribution to the publications

Peter McGranaghan contributed the following to the below listed publications:

Publication 1: **McGranaghan P**, Saxena A, Rubens M, Radenkovic J, Bach D, Schleußner L, Pieske B, Edelmann F, Trippel TD. Predictive value of metabolomic biomarkers for cardiovascular disease risk: a systematic review and meta-analysis. Biomarkers. 2020 Feb 17;25(2):101-11.
Contribution: Study design, data collection, ensuring the data quality, data analysis, interpretation of data, preparation of all the figures and tables, writing the manuscript
Impact factor 2015: 2.016

Publication 2: **McGranaghan P**, Düngen HD, Saxena A, Rubens M, Salami J, Radenkovic J, Bach D, Apostolovic S, Loncar G, Zdravkovic M, Tahirovic E, Veskovic J, Störk S, Veledar, E, Pieske B, Edlemann F, Trippel T. Incremental prognostic value of a novel metabolite-based biomarker score in congestive heart failure patients. ESC heart failure. 2020 Oct;7(5):3029-39.
Contribution: Data analysis, interpretation of the data, preparation of all the figures and tables, writing the manuscript
Impact factor 2019: 3.902

Publication 3: **McGranaghan P**, Saxena A, Düngen HD, Rubens M, Appunni S, Salami J, Veledar E, Lacour P, Blaschke F, Obradovic D, Loncar G, Apostolovic E, Edelmann F, Pieske B, Trippel T. Performance of a cardiac lipid panel compared to four prognostic scores in chronic heart failure. Sci Rep 11, 8164 (2021).
Contribution: Data analysis, interpretation of the data, preparation of all the figures and tables, writing the manuscript
Impact factor 2019: 3.998

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

10.1 Publication 1

McGranaghan P, Saxena A, Rubens M, Radenkovic J, Bach D, Schleußner L, Pieske B, Edelmann F, Trippel TD. Predictive value of metabolomic biomarkers for cardiovascular disease risk: a systematic review and meta-analysis. Biomarkers. 2020 Feb 17;25(2):101-11.

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10.2 Publication 2

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ORIGINAL RESEARCH ARTICLE

Incremental prognostic value of a novel metabolite-based biomarker score in congestive heart failure patients

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Abstract

Aims The Cardiac Lipid Panel (CLP) is a newly discovered panel of metabolite-based biomarkers that has shown to improve the diagnostic value of N terminal pro B type natriuretic peptide (NT-proBNP). However, little is known about its usefulness in predicting outcomes. In this study, we developed a risk score for 4-year cardiovascular death in elderly chronic heart failure (CHF) patients using the CLP.

Methods and results From the Cardiac Insufficiency Bisoprolol Study in Elderly trial, we included 280 patients with CHF aged >65 years. A targeted metabolomic analysis of the CLP biomarkers was performed on baseline serum samples. Cox regression was used to determine the association of the biomarkers with the outcome after accounting for established risk factors. A risk score ranging from 0 to 4 was calculated by counting the number of biomarkers above the cut-offs, using Youden index. During the mean (standard deviation) follow-up period of 50 (8) months, 35 (18%) subjects met the primary endpoint of cardiovascular death. The area under the receiver operating curve for the model based on clinical variables was 0.84, the second model with NT-proBNP was 0.86, and the final model with the CLP was 0.90. The categorical net reclassification index was 0.25 using three risk categories: 0–60% (low), 60–85% (intermediate), and >85% (high). The continuous net reclassification index was 0.772, and the integrated discrimination index was 0.104.

Conclusions In patients with CHF, incorporating a panel of three metabolite-based biomarkers into a risk score improved the prognostic utility of NT-proBNP by predicting long-term cardiovascular death more precisely. This novel approach holds promise to improve clinical risk assessment in CHF patients.

Keywords Metabolomics; Metabolite profiling; Prognosis; Biomarkers; Congestive heart failure

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Introduction

Chronic heart failure (CHF) is a leading cause of morbidity and mortality. Its prevalence continues to rise in developed countries, partly because of a shift in the age distribution of the population and improved treatment and care.[1,2] Clinicians should ensure that patients with CHF have the necessary knowledge and resources to make the best health decisions. Accurate and improved decision support methods, such as tools to predict the risk of mortality and prognosis of

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patients, could help in making such shared decisions for treatment plans and risk management strategies. Recently, there has been an increase in the number of prognostic biomarkers being tested for CHF such as growth differentiation factor-15,[3,4] high-sensitivity C-reactive protein,[5,6] galectin-3,[7–9] and high-sensitivity troponin T.[10,11] However, the added value of these markers is still under debate, and long-term follow-up studies are lacking. Among biomarkers widely used in CHF, N terminal pro B type natriuretic peptide (NT-proBNP) is recognized as a standard reference for diagnosis and prognosis. Despite the clinical utility of NT-proBNP, some studies have reported a high intraindividual variance and high reference change values among patients with CHF.[12–14]

Metabolomic profiling, or metabolomics, can help meet the need for more robust prognostic biomarkers. This approach provides a holistic signature of biochemical activities in humans by detecting and quantifying low-weight molecules (<1500 Da) that could be associated with disease progression.[15–17] Studies of predictive metabolomic biomarkers in CHF have been published previously that support the overall hypothesis that circulating metabolites may be used for risk assessment of cardiovascular (CV) disease patients[18-20,21-29]. These studies appear promising, but validation and the additive value of these biomarkers are less established.

In a discovery phase untargeted metabolomic study by Mueller et al., comparing CHF patients to healthy controls, a novel panel of metabolites known as the Cardiac Lipid Panel (CLP) was found to improve the diagnostic performance over NT-proBNP alone.[30] Its prognostic performance, however, is unknown. Details of the CLP have been published previously.[30] In brief, the CLP is a biomarker panel consisting of three specific metabolomic features: triacylglycerol (TAG) 18:1/18:0/18:0, phosphatidylcholine (PC) 16:0/18:2, and the sum of the three isobaric sphingomyelin (SM) species SM d18:1/23:1, SM d18:2/23:0, and SM d17:1/24:1.

In this study, the prognostic value of the CLP was analysed in elderly patients with CHF. We developed a risk score for predicting 4-year CV death using cut-offs for the CLP, which improved predictive value of the standard reference biomarker, and traditional risk factors.

Materials and methods

Study population

The study sample was randomly selected from the Cardiac Insufficiency Bisoprolol Study in Elderly (CIBIS-ELD) trial, a multicentre, randomized, double-blind trial with ≥65-year-old patients with stable CHF. Details of the CIBIS-ELD trial have been published previously.[31,32] In brief, elderly patients with CHF were randomized in a 1:1 fashion to receive either bisoprolol or carvedilol, up titrated every 2 weeks for 12 weeks and then followed for 4 years. We only considered the baseline and 4-year follow-up time points for this study. From the 589 subjects with available blood samplesfrom CIBIS-ELD trial, patients were randomly selected and studied in a case cohort design. Following random selection, the cohort was filtered down based on the feasibility of performing the biomarker test, for instance if there was sufficient quantity of blood aliquot sample available for analysis, and whether blood samples passed quality assurance[33], resulting in a final set of 280 cases. The investigation conformed to the principles outlined in the Declaration of Helsinki.[34] The ethics committees of all participating centres approved the study protocol, and informed consent was signed by all participants prior to study participation.

Metabolite profiling

The serum samples were collected in 2006–2007 at the time of the CIBIS-ELD study initiation, stored at −80°C, and then shipped on dry ice in 2014 to the metabolomics lab for analysis. Metabolite profiling of the serum samples was performed using a kit developed for the routine measurement of the CLP. The dedicated protocol was designed for utilization in the clinical practice setting and based on a one-phase extraction of the samples using gas chromatography mass spectrometry (GC-MS), followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis as previously described.[30] Sample and metabolite analysis quality assurance is part of the analytical protocol, so the metabolomic data that did not pass quality assurance were not included in this study[33]. The three CLP metabolomic features were generated at baseline, only for the previously mentioned samples.

Statistical analysis

Continuous variables were expressed as mean (standard deviation) and compared using *t*-test or Mann–Whitney *U* test, according to normal or non-normal distribution. Categorical variables were expressed as number (%). Comparisons among variables with more than two categories were performed using Wilcoxon rank sum test for continuous variables and Pearson's χ^2 test (or Fisher's exact test) or Mantel–Haenszel χ^2 test for categorical and ordinal data, respectively. All continuous predictor variables were log transformed to allow for direct comparison. Survival time was calculated from baseline until CV death or censoring at 4-year follow-up. Univariate Cox regression was performed on the CLP components and NT-proBNP, and multivariate Cox regression was also performed to adjust for clinical covariates. The considered clinical covariates were age, sex, body mass index, New York Heart Association class, creatinine, LDL cholesterol, triglycerides, left ventricular ejection fraction (LVEF), history of

diabetes, history of myocardial infarction, smoking history, hypertension, hyperlipidaemia, coronary artery disease, and medication including beta-blockers, aldosterone receptor blockers, angiotensin-converting enzyme inhibitors, anti-arrhythmic agents, aspirin, calcium channel blockers, diuretics, glycosides, nitrates, statins, sedative agents, and vitamin K antagonists. Hazard ratios and 95% confidence intervals were calculated for each univariate and multivariate model. The survival function for each model was generated using the predicted risk estimates following Cox regression.

To evaluate the predictive value of the CLP, three multivariable prediction models were built using Cox regression. The first model was built using the baseline clinical covariates only (Model A). Then, NT-proBNP was added to the first model (Model B). Finally, the CLP risk score was added to Model B (Model C). The CLP risk score was calculated as the sum of biomarkers above the Youden index cut-off.[35] There were four cut-off values, because four biomarkers are included in the score, three from the CLP and one from NT-proBNP. Each cut-off was calculated using Youden's index of the predicted probability from the Cox multivariate regression. Supplemental Data (*Data S1*) shows the equation for calculating the Youden cut-off. Based on the Youden cut-off, a value of 1 or 0 was assigned if the biomarker was above/below the cut-off value. A value of 1 was assigned in the direction of higher risk, that is, if a biomarker was protective (hazard ratio < 1), then a 1 value was assigned if the biomarker was below the Youden cut-off and vice versa. Then, all four values were summed to generate the final score for each subject. To measure the discrimination of each model, the area under the receiver operating curve (AUROC)and Harrell's concordance statistics were calculated for the 4-year survival of Models A, B, and C. Differences in Uno's concordance statistics were calculated for hypothesis testing of the change in AUROC of the three models.[36]

To measure risk reclassification, both continuous and categorical net reclassification indexes (NRIs) were calculated as well as integrated discrimination improvement (IDI).[37,38] The categorical NRI used three categories of <60%, 60–85%, and >85% corresponding to low, intermediate, and high risk, respectively. The continuous NRI does not depend on the choice of categories, but allocates any change in predicted risk in the correct direction.[39] IDI measures the ability of the new model to increase average sensitivity without reducing average specificity.

For sensitivity analysis, we performed logistic regression in addition to the Cox regression analysis, using the same independent and dependent variables in order to assess whether a different statistical model would yield similar results. We also tested two additional outcomes: the first was major adverse CV events defined as either myocardial infarction, transient ischaemic attack, stroke, or CV mortality, and the second outcome was all-cause mortality. Comparison of receiver operating curves following logistic regression was done using the Mann–Whitney *U* test.To test the sensitivity of NRI variation in risk categories, we used the same number of risk categories ($n = 3$) but readjusted the cut-off values using two separate sets of cut-offs, which still corresponded to high, medium, and low. The first set was 70% and 90% followed by the second set of 80% and 95%. Statistical analysis was performed using SAS software version 9.4 and R software version 3.6.1.[400–42]

Results

Baseline characteristics

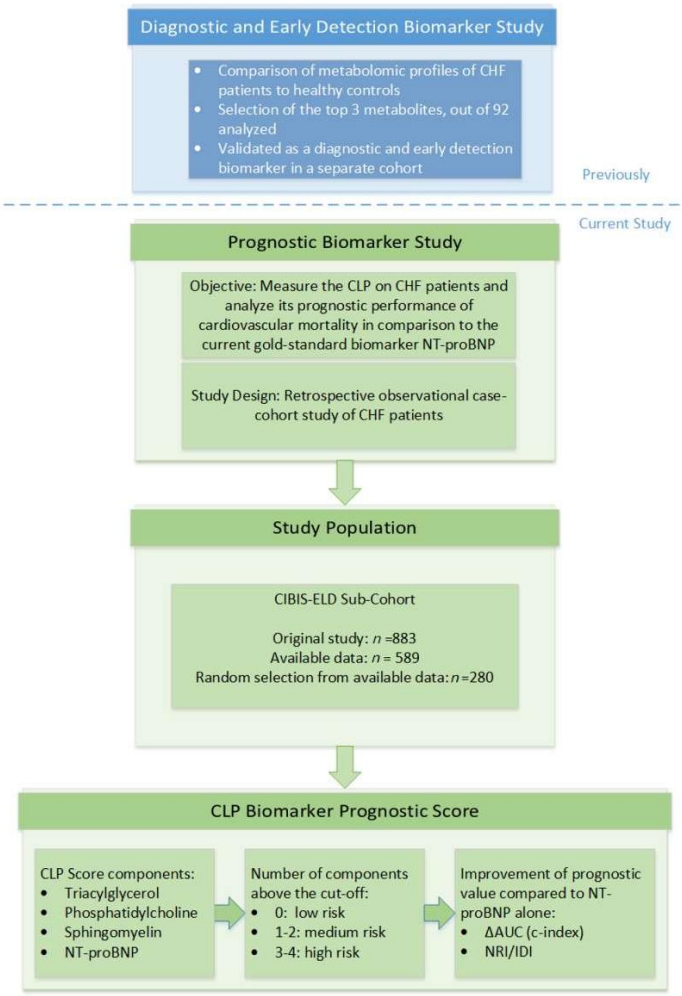
Figure 1 shows the study rationale and selection of subjects for this subcohort. The previously discovered CLP metabolites found to improve diagnosis of CHF were studied to assess their prognostic value. *Table 1* shows the baseline characteristics of the subsample population ($n = 280$) with a comparison to the source CIBIS-ELD cohort ($n = 589$). Mean patient age was 72.1 (4.9) years, 73.6% were men, 45% patients had heart failure with reduced ejection fraction (LVEF $<$ 35%), 49% had heart failure with mid-range ejection fraction (LVEF 35–49%), 4% had heart failure with preserved ejection fraction (LVEF $\geq$ 50%), and the majority of patients were in New York Heart Association functional class II (67.5%). During the follow-up period (mean = 50 months, standard deviation = 8; median = 46 months), 35 (13%) died from CV causes.

Prognostic performance and risk reclassification

Table 2 shows the univariate (unadjusted) and multivariate (adjusted) models of the CLP risk score components. In the unadjusted model, two of the three CLP biomarkers (PC and SM) were significantly associated as well as NT-proBNP. In the adjusted model, the same two CLP biomarkers remained significant but NT-proBNP did not. Supporting Information, *Table S1* shows the the hazard ratios for the clinical variables included in the adjusted model. *Figure 2* shows the measures of discrimination (AUROC) for the three multivariable models with a comparison to its preceding model to test the level of significance after adding the respective covariate(s), and Supporting Information, *Table S2* shows Harrell's concordance statistics. The AUROC for Model A was 0.84, that of Model B was 0.86, and the final adjusted Model C was 0.90. The difference in AUROC after adding the CLP score (Model B vs. C) was significant ($P = 0.02$), whereas the difference after adding NT-proBNP to the clinical model (Model A vs. B) was insignificant ($P = 0.47$).

Figure 3 shows the number of subjects reclassified into each risk category for Model A to B to C. *Table 2* shows the risk reclassification of Models B and C as percentages of total

FIGURE 1 Study rationale for the prognostic biomarker study. AUC, area under the curve; CHF, chronic heart failure; CIBIS-ELD, Cardiac Insufficiency Bisoprolol Study in Elderly; CLP, Cardiac Lipid Panel; IDI, integrated discrimination improvement; NRI, net reclassification index; NT-proBNP, N terminal pro B type natriuretic peptide.



with events and non-events. Supporting Information, *Table S1a–b* shows the frequency of cases per risk category stratified by events and non-events from Models B and C. The overall categorical NRI was 0.25 using the three risk categories 0–60%, 60–85% and >85%, meaning 25% of the subjects were reclassified into the respective correct risk category after adding the CLP (*Table 3*). Accordingly, 59% of the reclassified cases were downgraded, and the other 41% were upgraded. Among patients experiencing events, the overall categorical NRI was 0.60, with 33% of those downgraded and 67% upgraded. For non-events, the categorical NRI was 0.19, with 70% of those downgraded and 30% upgraded. The overall continuous NRI was 0.472 and the IDI was 0.019. The CLP model (Model C) showed that its high-risk

Table 1 Baseline characteristics of the study participants compared with the source cohort

Characteristic	*n* = 280	*n* = 589	*P* value
Age (years), mean ± SD	72 ± 4.9	72 ± 4.9	0.4190[a]
NYHA (II/III), *n*	188/91	374/183	0.5424[c]
Male, *n* (%)	206 (74)	412 (71)	0.1389[b]
Body mass index (kg/m^2), mean ± SD	26.8 ± 3.4	26.9 ± 3.9	0.4296[a]
Heart rate (bpm), mean ± SD	73 ± 13	74.7 ± 14	0.0031[a]
Systolic blood pressure (mm Hg), mean ± SD	134 ± 19	134 ± 19	0.2490[a]
Diastolic blood pressure (mm Hg), mean ± SD	81 ± 11	81 ± 11	0.3402[a]
Laboratory, mean ± SD			
Serum creatinine (μmol/l)	106 ± 29	107 ± 43	0.0096[a]
Haemoglobin (g/dL)	24.4 ± 34.8	14 ± 2	0.0325[a]
Sodium (mmol/L)	141.4 ± 3.3	141 ± 6.9	0.0765[a]
Uric acid (μmol/L)	273.2 ± 196.4	343 ± 121	0.0218[a]
Cholesterol (mmol/L)	5.5 ± 1.4	5.5 ± 1.4	0.2743[a]
HDL cholesterol (mmol/L)	1.2 ± 0.5	1.2 ± 0.5	0.4051[a]
LDL cholesterol (mmol/L)	3.4 ± 1.3	3.4 ± 1.2	0.348[a]
Triglycerides (mmol/L)	1.7 ± 1.0	1.8 ± 1.1	0.0283[a]
NT-proBNP (pg/mL)	793 (331–1765)[d]	873 (350–1931)[d]	0.0485[a]
Cardiac imaging, mean ± SD			
LVEF (%)	36 ± 9.5	37 ± 9.6	0.0899[a]
LVDed (mm)	58.8 ± 9.2	59.8 ± 9.3	0.0082[a]
LVDes (mm)	45.5 ± 9.7	46.5 ± 10.2	0.0089[a]
LVVed (mL)	152.7 ± 63.9	159 ± 67.7	0.0344[a]
LVVes (mL)	101.1 ± 51.6	105 ± 54.1	0.0705[a]
LAes (mm)	45.3 ± 7.2	45.2 ± 7.2	0.453[a]
E/e'	8 ± 4.3	11.1 ± 8.5	0.0025[a]
E/A	1 ± 0.8	1.1 ± 0.9	0.2928[a]
Deceleration time (ms)	226 ± 80	225 ± 79	0.7198[a]
Comorbidities, *n* (%)			
Diabetes	82 (29)	146 (25)	0.023[b]
Hypertension	224 (80)	469 (80)	0.7941[b]
Coronary artery disease	200 (71)	392 (67)	0.0382[b]
Smokers	125 (45)	257 (44)	0.7933[b]
Hyperlipidaemia	162 (58)	343 (59)	0.6822[b]
Medication, *n* (%)			
ACE inhibitor	247 (88)	509 (87)	0.527[b]
ARB	115 (41)	240 (41)	0.9643[b]
Glycoside	59 (21)	101 (17)	0.0216[b]
Aspirin	216 (77)	433 (74)	0.1273[b]
Nitrate	146 (52)	253 (43)	0.001[b]
Anti-arrhythmic agent	42 (15)	88 (15)	0.9512[b]
Statin	114 (41)	231 (40)	0.6044[b]

P values are compared with the available 589 subjects from the CIBIS-ELD cohort, which included this cohort of 280 subjects.
ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; E/A, ratio of the early (E) to late (A) ventricular filling velocities; E/e', ratio between early mitral inflow velocity and mitral annular early diastolic velocity; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NT-proBNP, N-terminal pro–B-type natriuretic peptide; NYHA, New York Heart Association; LAes, left atrial end systole; LVEF, left ventricular ejection fraction; LVDed, left ventricular diameter end diastole; LVDes, left ventricular diameter end systole; LVVed, left ventricular volume end diastole; LVVes, left ventricular volume end systole.
[a]Wilcoxon rank sum test.
[b]Pearson's χ^2 test.
[c]Mantel–Haenszel χ^2.
[d]Median (interquartile range).

category contained predominantly subjects who experienced an event (77%), whereas the respective fraction in the NT-proBNP model (Model B) was only 42%.

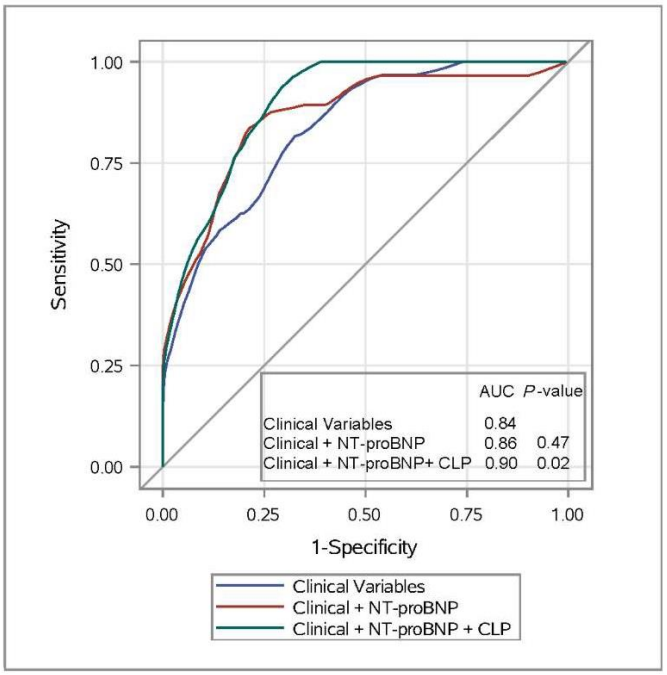
Results were consistent in the sensitivity analysis using logistic regression. We found that the differences in AUROC values pointed in the same direction as the Cox regression models (Model C AUROC = 0.90 vs. Model B AUROC = 0.86, *P* = 0.02). The change in AUROC after adding NT-proBNP to the clinical model (Model A AUROC = 0.84) remained insignificant (*P* = 0.47; Supporting Information, *Figure S1*). We found similar results when testing the models using the two additional outcomes, major adverse CV event and all-cause mortality (Supporting Information, *Figures S2* and *S3*). Readjustment of risk categories using the two different sets of cut-offs also showed similar results with the original set of cut-off values. The overall NRI for the first set of 70% and 90% cut-offs was 0.28 (Supporting Information, *Table S6*), and the second set of 80% and 90% cut-offs was 0.36 (Supplemental Information, *Table S7*). In addition, Model C was still able to classify a higher proportion of cases with events in the high-risk group than Model B in each scenario (Supporting Information, *Tables S3–SS5*).

Table 2 CLP risk score components and HRs

CLP component	Unadjusted HR (95% CI)	*P* value	Adjusted HR (95% CI)	*P* value
SM	0.36 (0.16–0.82)	0.0143	0.18 (0.04–0.76)	0.0039
PC	0.76 (0.64–0.89)	0.0007	0.53 (0.38–0.75)	0.0003
TAG	0.69 (0.47–1.02)	0.0644	0.67 (0.35–1.25)	0.2069
NT-proBNP	1.49 (1.12–1.99)	0.007	1.60 (0.975–2.625)	0.0630

Adjusted Cox proportional hazard model considers the following clinical covariates: age, sex, body mass index, New York Heart Association class, creatinine, LDL cholesterol, triglycerides, left ventricular ejection fraction, history of diabetes, history of myocardial infarction, smoking history, hypertension, hyperlipidaemia, coronary artery disease, beta-blockers, aldosterone receptor blockers, angiotensin-converting enzyme inhibitors, anti-arrhythmic agents, aspirin, calcium channel blockers, diuretics, glycosides, nitrates, statins, sedative agents, and vitamin K antagonists.
CI, confidence interval; CLP, Cardiac Lipid Panel; HR, hazard ratio; NT-proBNP, N terminal pro B-type natriuretic peptide; SM, sum of the 3 isobaric sphingomyelin species: SM d18:1/23:1, SM d18:2/23:0, and SM d17:1/24:1; PC, phosphatidylcholine 16:0/18:2; TAG, triacylglycerol 18:1/18:0/18:0.

FIGURE 2 Discrimination analysis of the CLP biomarker risk score for 4-year cardiovascular mortality. AUC, area under the curve; CLP, Cardiac Lipid Panel; NT-proBNP, N terminal pro B type natriuretic peptide.



Discussion

In this post hoc analysis of the CIBIS-ELD trial analysing 280 elderly patients with CHF, we showed that a risk score based on a novel panel of metabolites added prognostic value for the prediction of long-term CV mortality. A previous study already had reported that the CLP may improve the early detection and diagnosis of CHF.[30] However, to the best of our knowledge, the current study is first to estimate the prognostic performance of the CLP. Risk prediction models allow clinicians to accurately assess patient prognosis and facilitate more effective risk stratification and, ideally, a personalized treatment. Devising a more accurate biomarker panel for CHF risk prediction may aid clinicians with the difficult

FIGURE 3 Risk reclassification of subjects after adding N terminal pro B type natriuretic peptide to the clinical model followed by adding the Cardiac Lipid Panel biomarker score.

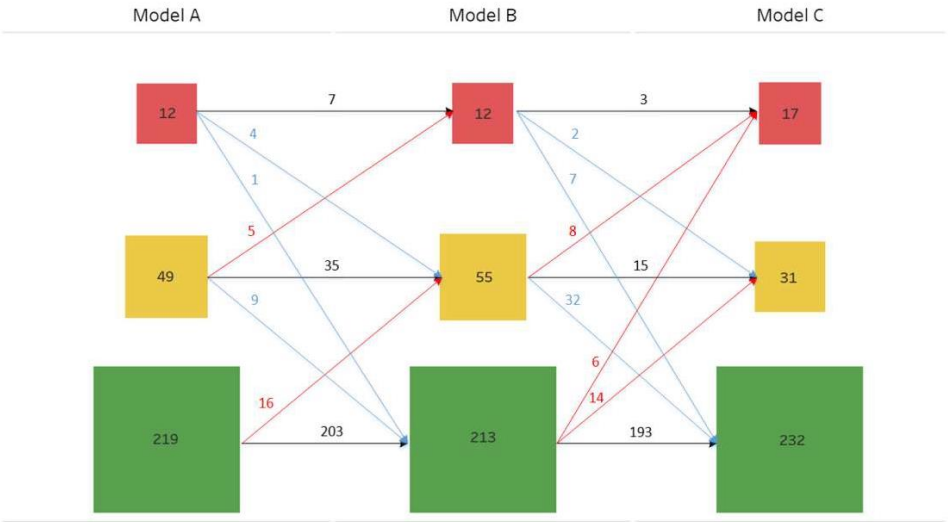


Table 3 Risk reclassification of total subjects, cases, and non-cases after adding the CLP risk score to the NT-proBNP based model

		Model B			
	Risk category	Low	Medium	High	Total
Model C	Low	69% 96%, 4%	12% 84%, 16%	2% 71%, 29%	83%
	Medium	5% 71%, 29%	5% 80%, 20%	1% 100%, 0%	11%
	High	2% 33%, 67%	3% 25%, 75%	1% 0%, 100%	6%
	Total	76%	20%	4%	100%

Percentage of subjects within each risk category of each Model A and B only. Events and non-events are proportions of the group total and are comma separated with red denoting events and blue denoting non-events.
Model B is the clinical covariates + NT-proBNP, Model C is clinical covariates + NT-proBNP + CLP score. Total subjects, $n = 280$; total events, $n = 35$.
The considered clinical covariates were age, sex, body mass index, New York Heart Association class, creatinine, LDL cholesterol, triglycerides, left ventricular ejection fraction, history of diabetes, history of myocardial infarction, smoking history, hypertension, hyperlipidaemia, coronary artery disease, and medication including beta-blockers, aldosterone receptor blockers, angiotensin-converting enzyme inhibitors, anti-arrhythmic agents, aspirin, calcium channel blockers, diuretics, glycosides, nitrates, statins, sedative agents, and vitamin K antagonists.
Categorical net reclassification index was calculated according to risk cut-offs of <60%, 60–85%, and >85% corresponding to risk categories low, medium, and high, respectively.

decisions surrounding the management of such high-risk patients. Conversely, identifying patients at lower risk may help reassure both clinicians and patients.

In the current study, two out of the three CLP components as well as NT-proBNP were independently associated with the outcome, so our next step was to build a risk score using these four components. The CLP risk score showed improved discrimination and risk reclassification in comparison with NT-proBNP alone, which is the current reference standard. Adding NT-proBNP to the clinical model slightly but insignificantly improved discrimination, while adding the CLP yielded a significant change in AUROC. Risk reclassification was improved by adding the CLP as it correctly identified a higher proportion of high-risk patients experiencing an event. For non-events, the majority of reclassified cases (70%) were downgraded. This indicates that added information of CLP also aided the proper classification of low-risk patients. Although both Models B and C misclassified some patients who did not experience any event in the high-risk group, the model with CLP had higher specificity as it classified

35% more patients with an event into the high-risk group. The continuous NRI also showed that Model C, compared with Model B, produced higher (i.e. more accurate) risk estimates for patients experiencing an event and lower risk estimates for those who are not.

Application of a single biomarker such as NT-proBNP for outcome prediction is primarily limited by insufficient specificity, resulting in a high false positive rate or low positive predictive value.[43,44] Because NT-proBNP is really a marker of elevated atrial pressures and volume overload, it may be of limited use in well-compensated, clinically stable heart failure patients. Hence, supporting this marker at the metabolic level may provide additional prognostic value and potentially phenotypic information.A combination of several metabolomic features into a biomarker panel or a risk score may provide a better prognosis utility over single biomarkers. A systematic review[21] reported that 6 out of 12 articles[22–27] developed a score by combining between 4 and up to 16 metabolites to predict CV risk. Recently, Lanfear et al. identified and then validated a panel of 13 circulating metabolites as a predictor of mortality risk in HF patients after accounting for conventional clinical risk factors and NT-proBNP levels.[28] Another prospective population-based study deriving a risk score from four metabolites and validating this score in two cohorts found improved risk reclassification of CHF patients using the biomarker score, although discrimination was not significantly enhanced.[29] A meta-analysis of 18 metabolomic prediction studies of CV disease outcomes reported an average change in c-statistic of 0.0417 (standard error 0.008) after adding metabolite-based information, which is consistent with our results. Of note, the metabolite score subgroup performed best (n = 5 studies),[45] although publication bias and heterogeneity were reported regarding variations in cohorts, study design, and metabolite profiling approaches.

In addition to investigating the improvement of the prognostic performance of CV outcomes, it is conceivable that metabolomic findings may also foster a better understanding of the pathophysiology and biological mechanisms involved in the development of CHF events. Altered lipid metabolism and dyslipidaemia are known to be associated with inflammation and oxidative stress, which are primary drivers of the pathological changes in CHF. The CLP metabolites belong to three different lipid classes, sphingomyelin (SM) phosphatidylcholine (PC), and triglycerides (TAG), and may be involved in different dysregulated metabolic pathways in CHF such as cell stress, inflammation, and atherosclerosis, although future studies are needed to assess whether the CLP biomarkers are representative of altered biological pathways. It has been previously shown that pathway-specific biomarkers/scores consisting of high-sensitivity C-reactive protein (inflammation), soluble urokinase plasminogen activator receptor (inflammation), fibrin degradation products (thrombosis), and heat shock protein 70 (cell stress) significantly improved the prediction of adverse cardiac events in high-risk populations. These studies also reported similar increases in c-statistics as this study after adding the pathway-specific biomarkers to predictive models.[46–48]

The combination of the CLP's metabolomic features with NT-proBNP may help overcome well-known limitations of NT-proBNP regarding clinical risk factors like age, gender, body mass index, and LVEF. A strength of this study is the high mean age, because elderly patients are underrepresented in CHF trials although CHF is responsible for a great deal of morbidity and mortality in the aging population.[49] Moreover, study samples were derived from a well-characterized cohort including high-quality assessment of outcome events. In future studies, we would like to further elucidate the prognostic utility of the CLP and externally validate its clinical effectiveness by including a larger cohort with more women and patients with early stage CHF and testing different biological matrices (e.g. plasma). Following these studies and regulatory approval, it is conceivable that this biomarker panel can be tested alongside the standard NTpro-BNP test in the clinical setting for a more precise risk assessment of CHF patients (Supporting Information, *Figure S4*).

Study limitations

Our findings can only be interpreted in the context of this specific subcohort and the CLP metabolites, which limits the generalizability of our findings. We were limited by the ability to perform the CLP analysis on separate cohorts, but these proof of concept data can be used as a reference point for additional and larger validation cohorts in the future. The CLP was originally discovered and intended as a diagnostic biomarker, and we cannot assume that it is also a powerful prognostic algorithm as these are still preliminary findings needing validation. Ideally, a prospective derivation validation design using an untargeted metabolite profiling approach should be used to discover a novel prognostic biomarker; however, we were limited on available data and resources. Our findings can only be interpreted as exploratory.

The sample selection criteria, based on the availability and quality of blood samples, may have introduced selection bias for subjects who were more willing or prone to have blood withdrawn and may have excluded patients who were not able to provide sufficient blood possibly due to other CV risk factors, socio-economic status, or comorbidities. The serum samples used in this study may have been affected by the long-term storage prior to the CLP assay, as lipid parameters are known to be subject to in vitro degradation. The quality assurance methods used in this CLP protocol only apply to the sample preparation and measurement requirements for the identification and analysis of the CLP features and do not adjust for any potential effects of prolonged storage. Although NT-proBNP is the gold standard biomarker for CHF

patients, we did not find a significant increase in prognostic power after adding this biomarker to the clinical model, possibly due to the homogeneity of our population of elderly, stable CHF patients, in which it may be of limited use given the fact that NT-proBNP is a marker of volume overload and elevated atrial pressures. Other common cardiac biomarkers, such as troponins or C-reactive protein, should also be evaluated for their incremental prognostic power because a more comprehensive biomarker profile for prognosis may be a better solution than including only CLP plus NT-proBNP.

The samples from the population in this study may have been affected by other medications or a combination of comorbidities that can affect the lipid metabolites in the CLP. The cut-off values used to generate the CLP risk score using Youden's index are specific to this cohort and not universally applicable, as a large validation cohort(s) would be required to create a generalized equation that could be used in the daily routine management of CHF. The NRI as well as the IDI can be affected by the event rate, which is low in our study.. Although all biomarkers were log transformed, they were not normally distributed, which could affect the concordance of the NRI and IDI. The choice of cut-offs for categorical NRI to determine incremental predictive performance was challenging, as there seems to be no standardized guideline for choosing NRI cut-offs. We found that the NRI was sensitive to changes in the definition of risk categories; however, results did not differ in the sensitivity analysis.

Although this biomarker was developed for routine clinical use, it is currently only available in specialized labs equipped with mass spectrometry equipment.While the CLP is still a research tool awaiting further translation to the routine lab, as an ELISA test for example, this study represents the first step towards that direction.

Conclusions

Our findings demonstrate that the CLP risk score comprising a panel of three lipid-based metabolomic features meaningfully improved the prediction of CV mortality and reclassified patients to their proper risk categories. This new panel of lipid metabolites may complement currently used biomarkers such as NT-proBNP. Thus, the metabolomics approach may potentially translate into clinical applications such as routinely applied risk stratification and targeted treatments for CHF patients.

Acknowledgements

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Conflict of interest

Charité—University Medicine Berlin holds the intellectual property under patents WO 2011092285, WO 2015028671, WO 2016034600, Means and methods for diagnosing heart failure in a subject, WO 2014060486, WO 2014060486, Means and methods for determining a clearance normalized amount of a metabolite disease biomarker in a sample, WO 2016016258 Means and methods for diagnosing heart failure on the basis of cholesterol parameters, sphingomyelins and/or triacylglycerols. CIBIS-ELD was supported by the German Federal Ministry of Education and Research (grant number 01GI0205). Sponsor according to ICH-GCP was the Charité—Universitätsmedizin (Berlin, Germany). Merck KGaA provided an unrestricted grant without any rights to influence trial design, data collection, data analysis, and interpretation or publication of CIBIS-ELD. The formerly existing Metanomics Health GmbH (Berlin, Germany) supported the presented analysis here by a research grant and performed the measurements without any rights to influence design, data collection, data analysis and interpretation, or publication of the current manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplemental equations.

Figure S1. *Sensitivity analysis of the discrimination performance of the CLP biomarker risk score for 4-year cardiovascular mortality using logistic regression.*

Figure S2. *Sensitivity analysis of the discrimination performance of the CLP biomarker risk score for Major Adverse Cardiovascular Events (MACE) using logistic regression.*

Figure S3. *Sensitivity analysis of the discrimination performance of the CLP biomarker risk score for all-cause mortality using logistic regression.*

Figure S4. *Proposed patient flow for testing the CLP in addition to NT-pro-BNP.*

Table S1. *Clinical variables and multivariable HRs.*

Table S2. *Harrell's Concordance Statistics for the discrimination of Models A, B, and C.*

Table S3. *(A) Frequency of events and non-events per risk category of Model B by cardiovascular mortality status using risk categories of 60%–85%. (B) Frequency of events and non-events per risk category*

10.3 Publication 3

McGranaghan P, Saxena A, Düngen HD, Rubens M, Appunni S, Salami J, Veledar E, Lacour P, Blaschke F, Obradovic D, Loncar G, Apostolovic E, Edelmann F, Pieske B, Trippel T. Performance of a Cardiac Lipid Panel Compared to Four Prognostic Scores in Chronic Heart Failure. Submitted to Scientific Reports.

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Performance of a cardiac lipid panel compared to four prognostic scores in chronic heart failure

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The cardiac lipid panel (CLP) is a novel panel of metabolomic biomarkers that has previously shown to improve the diagnostic and prognostic value for CHF patients. Several prognostic scores have been developed for cardiovascular disease risk, but their use is limited to specific populations and precision is still inadequate. We compared a risk score using the CLP plus NT-proBNP to four commonly used risk scores: The Seattle Heart Failure Model (SHFM), Framingham risk score (FRS), Barcelona bio-HF (BCN Bio-HF) and Meta-Analysis Global Group in Chronic Heart Failure (MAGGIC) score. We included 280 elderly CHF patients from the Cardiac Insufficiency Bisoprolol Study in Elderly trial. Cox Regression and hierarchical cluster analysis was performed. Integrated area under the curves (IAUC) was used as criterium for comparison. The mean (*SD*) follow-up period was 81 (33) months, and 95 (34%) subjects met the primary endpoint. The IAUC for FRS was 0.53, SHFM 0.61, BCN Bio-HF 0.72, MAGGIC 0.68, and CLP 0.78. Subjects were partitioned into three risk clusters: low, moderate, high with the CLP score showing the best ability to group patients into their respective risk cluster. A risk score composed of a novel panel of metabolite biomarkers plus NT-proBNP outperformed other common prognostic scores in predicting 10-year cardiovascular death in elderly ambulatory CHF patients. This approach could improve the clinical risk assessment of CHF patients.

The prevalence of chronic heart failure (CHF) in the western world continues to increase, especially in patients older than 65 years[1]. CHF is a major burden on the health care system and is associated with high morbidity and mortality, including a poor quality of life[2]. An important aspect of CHF management is to ensure that clinicians and patients with CHF have the necessary knowledge and resources to make the best health decisions. A prognostic model is one such resource, defined as a formal combination of multiple predictors from which risks of a specific outcome can be calculated for individual patients.

Prognostic models are abundant in the literature, and the most popular ones include the SHFM (Seattle Heart Failure Model), FRS (Framingham Risk Score), MAGGIC (Meta-analysis Global Group in Chronic Heart Failure), and BCN Bio-HF (Barcelona Bio-Heart Failure Risk Calculator). The SHFM score is the most thoroughly validated and contains the most predictor variables of the four[3]. The MAGGIC score[4] was developed from a dataset of over 39,000 patients across 30 studies and validated on more than 60,000 patients using 2 large CHF cohorts[5,6]. The FRS score was developed as a sex-specific risk score that can be conveniently used to calculate general cardiovascular disease (CVD) risk and risk of individual CVD events[7]. The BCN Bio-HF score contains 11

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clinical variables with the most biomarker variables [NT-proBNP, high-sensitivity cardiac troponin T (hs-cTnT), high-sensitivity soluble ST2 (ST2)] and has been externally validated[8,9]. These models all use common clinical and demographic variables to predict the prognosis of CHF patients and have convenient online calculators. Although these scores have been validated, they have not been widely adopted possibly because they are not routinely calculated in clinical practice[10–12], have poor reliability at the individual patient level[5], suffer from a significant amount of missing data requiring imputation.

Metabolomics is a rapidly growing field in biomarker profiling that could help meet the need for more robust prognostic biomarkers. By applying nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), it is now possible to analyze hundreds of metabolites from human samples such as blood, urine, saliva, and tissue, which can elucidate the outcome of complex networks of endogenous and exogenous biochemical reactions[13]. This approach could provide a more comprehensive signature of biochemical activities that could be associated with diet, medication, disease progression, and thus negative outcomes due to these complex mechanisms[14,15]. Previous studies have shown that metabolomic biomarkers can be used for risk prediction as well as diagnosis of CHF[16–28].

One promising metabolomic biomarker panel in CHF patients is the cardiac lipid panel (CLP) which is supplemented by N-terminal pro–B-type natriuretic peptide (NT-proBNP). The CLP is consists of three specific metabolomics features: triacylglycerol (TAG) 18:1/18:0/18:0, phosphatidylcholine (PC) 16:0/18:2, and the sum of the 3 isobaric sphingomyelin (SM) species SM d18:1/23:1, SM d18:2/23:0, and SM d17:1/24:1. The diagnostic value of CLP was first discovered in a study by Mueller and colleagues, where they compared CHF patients to healthy controls, and found that CLP was able to improve the diagnostic performance over NT-proBNP alone[29]. The incremental prognostic value of the CLP was first assessed in a recent study which found it improved the discrimination and risk assessment over NT-proBNP and clinical risk factors[30].

The objective of this study was to compare the performance of a risk score composed of the CLP panel plus NT-proBNP to the four commonly used traditional risk scores (SHFM, FRS, MAGGIC, BCN Bio-HF) to predict long-term cardiovascular mortality in ambulatory CHF patients. We hypothesized that the CLP risk score would improve our ability to classify risk of cardiovascular death in comparison to the four validated clinical risk prediction algorithms.

Results

Table 1 shows the baseline characteristics of the total population (n = 280) as well as the variables included in each score. Mean age of this sub-cohort was 72.1 (4.9) years, 26.4% were women, 45% patients had heart failure with reduced ejection fraction (HFrEF) (LVEF < 35%), and most patients were in NYHA functional class II (67.5%) with the remaining in NYHA class III. Hypertension was present in 80% of participants and 45% were current or former smokers; 29% had diabetes and 71% had CAD. During the follow-up period (mean = 81 months, SD = 33; median = 96 months), 95 (34%) patients met the primary outcome. There were 30 (11%) patients who met the secondary outcome of 3-year all-cause mortality. The sample selection criteria as well as the comparison of this sub cohort's baseline characteristics to the source cohort has previously been reported[30], however, this study analyzed 10 year follow up rather than the previously reported 4 year follow up.

All variables were available for each score except for the lymphocytes (%) variable in the SHFM score, which was imputed as previously described. The SHFM model had the highest number of variables (n = 17), followed by MAGGIC (n = 13), BCN Bio-HF (n = 12), FRS (n = 7), and CLP (n = 4). There were 13 overlapping variables which were included in at least 2 scores. The SHFM score included the most medication (n = 6) and laboratory (n = 5) variables, BCN Bio-HF is the only model with biomarker data (NT-proBNP) while MAGGIC included the most clinical (n = 7) and demographic variables (n = 3).

Table 2 shows the univariate Cox Regression results. The CLP (HR = 2.38, $p < 0.001$), SHFM (HR = 2.01, $p = 0.002$, MAGGIC (HR = 1.10, $p < 0.001$), and BCN Bio-HF (HR = 1.09, $p = 0.0393$) scores were significantly associated with the outcome while FRS was not. The hazard ratios for the secondary endpoint of 3-year all-cause mortality are shown in Supplemental Table 1. All scores had a higher HR than the primary outcome except for CLP and FRS. Figure 1 shows the AUC change over time (IAUC) for the 5 prognostic scores with the comparison of Uno's concordance statistics for hypothesis testing. The IAUC was 0.53, 0.61, 0.68, 0.72, and 0.78 for FRS, SHFM, MAGGIC, BCN Bio-HF, and CLP, respectively. Harrell's c statistics at 10 year follow up show similar results (Supplemental Table 2). The four traditional scores were all significantly different ($p < 0.05$) from the CLP score according to the difference in concordance statistic (Supplemental Table 3). The incremental value of the CLP to NT-proBNP is shown in Supplemental Figure 1, the NT-proBNP only IAUC was 0.71 while the CLP score (which incorporates the CLP biomarkers plus NT-proBNP) was 0.78 ($p = 0.004$). Discrimination analysis of the secondary outcome of 3-year all-cause mortality showed the CLP IAUC lowered to 0.76, and only CLP vs FRS remained significantly different (Supplemental Figure 2). The models showed adequate calibration except for FRS (calibration curve slope = 0.894) (Supplemental Figure 3).

Competing event analysis showed the SHFM, MAGGIC, and the CLP models remained significantly associated with cardiovascular death, and all scores showed less association to non-cardiovascular death (Supplemental Table 4). The CIF curve, which accounted for non-cardiovascular mortality as a competing event, showed higher cumulative incidence of cardiovascular mortality with higher CLP scores (Supplemental Figure 4).

Figure 2 shows the hierarchical cluster dendrogram mapped to illustrate the assignment of patients into their respective clusters and the associated color map shows the range of each prognostic score and their distribution within each cluster. Hierarchical clustering grouped the patients in separate clusters accounting for the noise between smaller clusters. Each observation was treated as a unique cluster, and this method: (1) identified the two similar or close clusters, and (2) merged the two most similar clusters. Using this clustering technique, similar prognostic score data from participants were grouped together, such that the members in the same group were

Characteristic	Total n = 280	Prognostic score SHFM	FRS	MAGGIC	BCN Bio-HF	CLP
Age (years), mean ± SD	72 ± 4.9	✓	✓	✓	✓	
NYHA (II/III), n	188/91	✓		✓	✓	
Male, n (%)	206 (74)	✓	✓	✓	✓	
Body mass index (kg/m^2), mean ± SD	26.8 ± 3.4			✓		
Heart rate (bpm), mean ± SD	73 ± 13.0					
Systolic blood pressure (mm Hg), mean ± SD	134 ± 19	✓	✓	✓		
Diastolic blood pressure (mm Hg), mean ± SD	81 ± 11					
Years since first diagnosis of CHF	5.2 ± 5.6		✓			
Laboratory, mean ± SD						
Creatinine (µmol/L)	107 ± 27.9			✓		
Hemoglobin (g/dL)	13.4 ± 1.5	✓			✓	
Sodium (mEq/L)	141.4 ± 3.3	✓			✓	
Uric acid (µmol/L)	356 ± 127	✓				
Total Cholesterol (mmol/L)	5.1 ± 1.6					
HDL cholesterol (mmol/L)	1.2 ± 0.5		✓			
LDL cholesterol (mmol/L)	3.4 ± 1.3					
Triglycerides (mmol/L)	1.7 ± 1.0					
Lymphocytes (%)*		✓				
NT-proBNP (pg/mL)	793 (331–1765)†				✓	✓
PC 16:0/18:2 (µg/dl)	36,810 (32,435–40,015)†					✓
TAG 18:1/18:0/18:0 (µg/dl)	121 (76.5–256.4)†					✓
SM d18:1/23:1, SM d18:2/23:0, SM d17:1/24:1 (µg/dl)	1342 (1134–1596)†					✓
Cardiac imaging, mean ± SD						
LVEF (%)	36 ± 9.5	✓		✓		
LVDed (mm)	58.8 ± 9.2					
LVDes (mm)	45.5 ± 9.7					
LVVed (mL)	152.7 ± 63.9					
LVVes (mL)	101.1 ± 51.6					
LAes (mm)	45.3 ± 7.2					
E/e'	12 ± 9.2					
E/A	1 ± 0.8					
Deceleration time (ms)	226 ± 80					
Comorbidities, n (%)						
Diabetes	82 (29)		✓	✓		
Hypertension	224 (80)					
Coronary artery disease	200 (71)	✓				
Smokers	125 (45)		✓	✓		
Hyperlipidemia	162 (58)					
COPD	9 (3)			✓		
Medication, n (%)						
ACE inhibitor	247 (88)	✓		✓		
Allopurinol	0 (0)	✓				
ARB	115 (41)	✓			✓	
Beta blocker	203 (73)	✓		✓		
Diuretics	219 (78)				✓	
Diuretic dose mg/kg per day	0.32 ± 0.31	✓				
Glycoside	59 (21)					
Aspirin	216 (77)					
Nitrate	146 (52)					
Antiarrhythmic agent	42 (15)					
Statin	114 (41)	✓			✓	

Table 1. Baseline characteristics of the study participants and variables included in each prognostic score. ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BCN Bio-HF, Barcelona Bio-Heart Failure Risk Calculator; CLP, Cardiac Lipid Panel Risk Score; COPD, chronic obstructive pulmonary disease; E/A, ratio of the early (E) to late (A) ventricular filling velocities; E/e', ratio between early mitral inflow velocity and mitral annular early diastolic velocity; FRS, Framingham Risk Score; LAes, left atrial end systole; LDL, low-density lipoprotein; NYHA, New York Heart Association; HDL, high-density lipoprotein; LVDed, left ventricular diameter end diastole; LVDes, left ventricular diameter end systole; LVVed, left ventricular volume end diastole; LVEF, left ventricular ejection fraction; LVVes, left ventricular volume end systole; mg/kg, milligrams per kilograms; MAGGIC, Meta-analysis Global Group in Chronic Heart Failure; NTpro-BNP, N-terminal pro–B-type natriuretic; PC, phosphatidylcholine; SHFM, Seattle Heart Failure Model; SM, sphingomyelin; TAG, triacylglycerol. *Imputed using the median, 31%, of the normal range 20–40%. †Median (Interquartile Range).

Score	HR (95% CI)	*p* value
SHFM	1.89 (1.29–2.807)	0.0017
FRS	1.02 (0.97–1.07)	0.5291
MAGGIC	1.10 (1.05–1.14)	<.0001
BCN Bio-HF	1.09 (1.00–1.84)	0.0393
CLP	2.38 (1.95–2.92)	<.0001

Table 2. Prognostic scores and univariate hazard ratios for cardiovascular mortality. Unadjusted Cox proportional hazard models of 10-year outcome for cardiovascular mortality. Total subjects, n = 280. Total events, n = 95. SHFM (Seattle Heart Failure Model), FRS (Framingham Risk Score), and MAGGIC (Meta-analysis Global Group in Chronic Heart Failure), BCN Bio-HF (Barcelona Bio-Heart Failure Risk Calculator), and Cardiac Lipid Panel Risk Score (CLP).

more similar to each other than the members in the other groups. We can infer from the cluster centres and cluster memberships that CLP risk score was better at grouping patients with respect to their cardiovascular mortality risk and associated clinical characteristics compared to the other four scores. The survival curves for each risk cluster are shown in Fig. 3. Rates of mortality were: low risk cluster (20%), moderate risk cluster (27%) and high-risk cluster (50%). Supplemental Figure 5 shows the constellation plot on a two-dimensional plane with nodes and links to describe relationship among component nodes. This plot is an alternate depiction of the dendrogram and illustrates the length between clusters and a balanced structure. Supplemental Figure 6 shows the scatterplot matrix of all 4 scores and clusters to illustrate the relationships between each prognostic score and risk cluster assignment.

Table 3 shows the cohort characteristics and the prognostic score distribution for each risk cluster. The three clusters were: low risk, n = 119; moderate risk, n = 44; high risk, n = 117. There were 11 out of the 50 cohort characteristics significantly different across the 3 clusters. In particular, patients in the highest risk cluster were older, with lower LVEF, higher NT-proBNP, and experienced a higher frequency of events. The SHFM, BCN Bio-HF, and CLP scoreswere significantly different across their respective risk clusters. Of the continuous risk scores (FRS, SHFM, MAGGIC, BCN Bio-HF), only SHFM and MAGGIC, had its highest mean score in the high-risk cluster. The categorical CLP score showed a skewed distribution of higher risk scores (3–4) in the moderate and high-risk clusters. In the high-risk cluster, the majority of subjects were scored with CLP scores of 3–4.

The correlation of the CLP biomarkers TAG, PC, and SM were most correlated with the clinical characteristics: triglycerides ($r = 0.531$, $p < 0.001$), total cholesterol ($r = 0.431$, $p < 0.001$), and LDL ($r = 0.502$, $p < 0.001$), respectively (Supplemental Figure 7).

Discussion

We found that a risk score based on a novel panel of three metabolite-based biomarkers plus NT-proBNP outperformed commonly used traditional prognostic models for predicting cardiovascular mortality in elderly ambulatory CHF patients. We first measured the association of each risk score with the outcome, followed by discrimination analysis, then cluster analysis, and finally correlation analysis of the individual CLP biomarkers with the clinical characteristics. In our study cohort, CLP score, showed the best discrimination compared to the other 4 scores. This indicates that the biomarker information included in the CLP score could more precisely classify high risk CHF patients than the information included in the 4 other risk scores. On the other hand, the biomarker information from the CLP is not as easily attainable and no convenient calculator exists yet, as these findings should first be validated in larger cohorts. Additionally, none of the other scores were originally developed for 10-year cardiovascular mortality. To the best of our knowledge there is no score specific for predicting 10-year risk of cardiovascular death, but it is not uncommon to use the scores such as FRS to predict different outcomes in similar studies[31–33]. Nevertheless, the other risk scores may be improved with the addition of common biomarkers in their score calculation. For instance, NT-proBNP is a well-established biomarker that is known to be associated with ventricular wall stress[34] and is considered the gold-standard biomarker in CHF diagnosis and prognosis[35]. Only BCN Bio-HF contained NT-proBNP and it was the next best performing prognostic score after the CLP.

We performed cluster analysis to assess how well the risk scores could partition subjects into different risk groups, blinded to the study outcome. A strength of this approach is that clusters could define relevant groups of patients and could mitigate the problems of multicollinearity while determining if the predictive variables are useful in separating these groups. In our study, patients within each cluster varied along measures of age, laboratory parameters, days survived, as well as the prognostic scores. When comparing the score distributions across the three risk clusters, the CLP score showed a more homogenous grouping of patients according to their risk score stratification while the other scores showed a more heterogenous distribution across risk clusters. Several prior studies have used similar clustering methods to identify clinically relevant patient subgroups for CHF[36,37], but we are not aware of previous studies using clustering methods to compare a novel biomarker score to other conventional prognostic scores for CHF.

The combination of the CLP's metabolomic features with NT-proBNP into a risk score may help overcome limitations of using only traditional clinical risk factors. Furthermore, application of a single biomarker such as NT-proBNP for outcome prediction is limited by insufficient specificity (low predictive value or high false positive rate)[38,39]. Recently, it was reported that the CLP added incremental prognostic value to NT-proBNP in

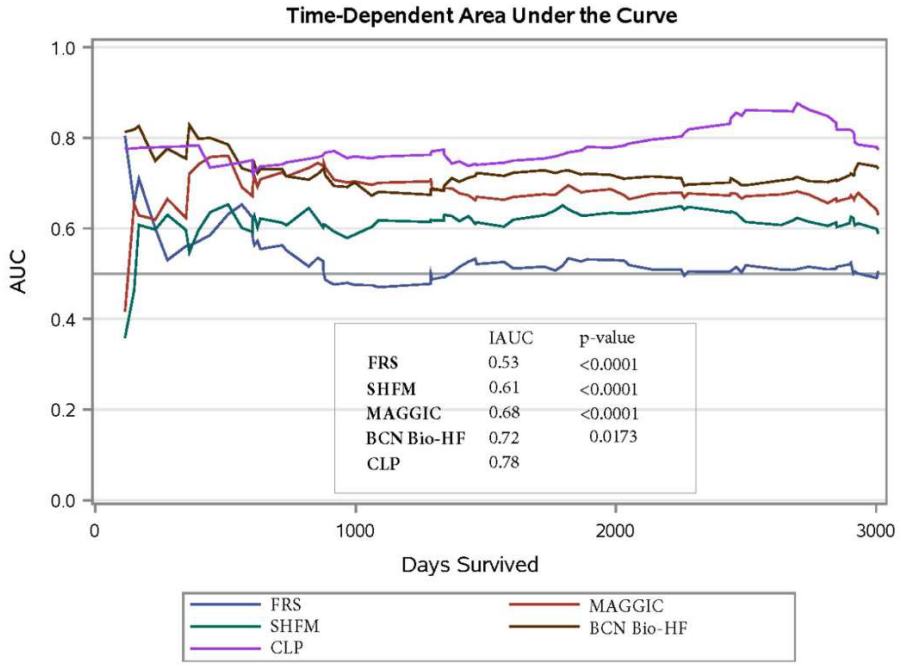

Figure 1. Discrimination performance for each prognostic score for 10-year cardiovascular mortality. Integrated area under the curve (IAUC) for: SHFM (Seattle Heart Failure Model), FRS (Framingham Risk Score), and MAGGIC (Meta-analysis Global Group in Chronic Heart Failure), BCN Bio-HF (Barcelona Bio-Heart Failure Risk Calculator), and Cardiac Lipid Panel Risk Score (CLP). Total subjects, n = 280; total events, n = 95. *p* values were calculated from the differences in concordance statistic in comparison to the CLP score.

predicting 4-year cardiovascular mortality[30]. We used the same method to calculate the CLP score for this study, and we also confirmed that the CLP provided similar incremental value to NT-proBNP alone as previously found in the 4-year study[30].Using an aggregate score rather than individual biomarkers for risk prediction can help more precisely stratify risk. A recent meta-analysis of 18 metabolomic prognostic biomarker studies for CVD found those which incorporated a selection of metabolites into a score (n = 5 studies) had the best prognostic performance rather than using the individual biomarker values[16]. Another systematic review[20] reported 6 studies[21–26] developed a metabolite-based score to predict CVD risk with each score composed between 4 and 16 biomarkers.

We have briefly mentioned the components of the CLP in the introduction section, in addition to improving risk prediction, developing a biomarker-based risk score could also improve our understanding of the pathophysiology and biological mechanisms involved in CHF. In the following paragraphs we would like to highlight those mechanisms based on previous research. The CLP metabolites can be grouped into three different lipid subclasses, sphingomyelin (SM) phosphatidylcholine (PC), and triglycerides (TAG), which have previously been found to be associated with cardiomyocyte stress/apoptosis[40], intestinal microbial metabolism/inflammation[19], and coronary artery disease[41], respectively. Sphingomyelins are localized in cell membranes and lipoproteins, and their hydrolysis by sphingomyelinase leads to increased amounts of ceramide. Ceramide triggers the generation of reactive oxygen species (ROS) involved in the modulation of cell proliferation and apoptosis, neutrophil adhesion to the vessel wall, and vascular tone. Dysfunctional sphingomyelin and ceramide metabolism may lead to or aggravate cardiovascular diseases[42]. Lemaitre et al.[43] reported that lipid species such as Cer-16 and SM-16 were associated with increased risk of heart failure. Sigruener et al.[44] reported that the detection of sphingomyelin species SM 16:0, 16:1, 24:1 and 24:2 was increasingly associated with mortality in Ludwigshafen Risk and Cardiovascular Health (LURIC) study. The CLP biomarker panel consists of the sum of three monosaturated fatty acid carrying SM species: SM d18:1/23:1, SM d18:2/23:0, SM d17:1/24:1.

PC is the most abundant lipid in the human body and is subjected to chemical events like lipid peroxidation and ROS formation[45]. Myocardium suffers heavily from lipid peroxidation related injury[46]. PC carrying polyunsaturated fatty acids such as PC (16:0/18:2) which is a component of the CLP panel, have increased risk for lipid peroxidation[47]. Oxidative stress increases the formation of electrophilic aldehydes from native phospholipids leading to formation of adducts with tissue or plasma proteins thereby aggravating the pathophysiology of cardiovascular diseases[48]. Previous studies have shown that lipid peroxidation and ROS generation are associated with cardiac damage and raises mortality. Higher consumption of PC was found to increase the risk of organ

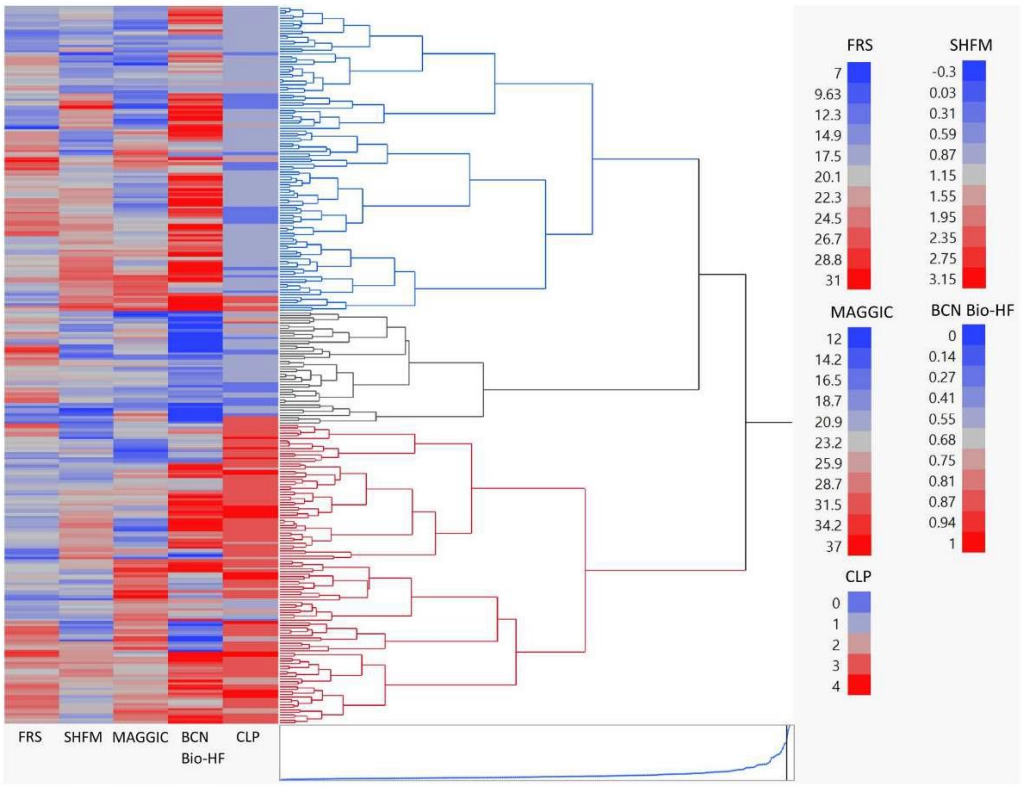

Figure 2. Hierarchal cluster dendrogram of three risk clusters. Assignment of patients into risk clusters based on the prognostic scores. The clustering process can be viewed by reading the dendrogram from left to right. Each step consists of combining the two closest clusters into a single cluster. The joining of clusters is indicated by horizontal lines that are connected by vertical lines. The horizontal position of the vertical line represents the distance between the two clusters that are most recently joined to form the specified number of clusters. The prognostic scores used for clustering were: SHFM (Seattle Heart Failure Model), FRS (Framingham Risk Score), and MAGGIC (Meta-analysis Global Group in Chronic Heart Failure), BCN Bio-HF (Barcelona Bio-Heart Failure Risk Calculator), and Cardiac Lipid Panel Risk Score (CLP). Each prognostic score was standardized to the same scale (mean = 0; SD = 1). Ward's minimum variance method was used for clustering. Blue dendrogram indicates the cluster 1 (low risk), n = 119; Grey dendrogram indicates cluster 2 (moderate risk), n = 44, Red dendrogram indicates cluster 3 (high risk), n = 117; Total subjects, n = 280.

injury and cardiovascular mortality[49]. Natural antioxidants like α-tocopherol have shown to reduce such oxidative stress and resulting inflammation thereby preventing the progression of cardiac injury[50].

The molecules of TG are involved in the regulation of insulin-signaling pathways through the activation of several serine/threonine kinases, which suppress insulin receptors, thus inducing peripheral insulin resistance. Previous studies have shown that insulin resistance leads to inflammation and atherosclerosis[51]. Although the relationship between total triglycerides and insulin resistance and CVD risk are well established[52], the relationships between individual serum TGs and insulin resistance is not well-established. Studies of individual TGs may help better characterize insulin resistance and CVD better than total triglycerides. For instance, it was previously found that saturated TG 16:0 fatty acid was positively associated with fasting serum insulin concentrations and that of essential 18:3 n-6 fatty acid was negatively associated[53]. Another study on individual TGs revealed that serum TG molecules containing saturated and monounsaturated fatty acids, such as TG(16:0/16:0/18:1) and TG(16:0/18:1/18:0), correlated positively, whereas those containing essential fatty acids, such as TG(18:1/18:2/18:2), negatively with features of insulin resistance[54]. The CLP consists of the saturated and monosaturated fatty acid carrying TAG 18:1/18:0/18:0.

These findings indicate that metabolomic studies may help gain a deeper understanding of the molecular mechanisms of CVD. Therefore, more detailed metabolomic analysis would hopefully lead to the discovery or further development of sensitive and specific lipid-based markers for cardiovascular risk.

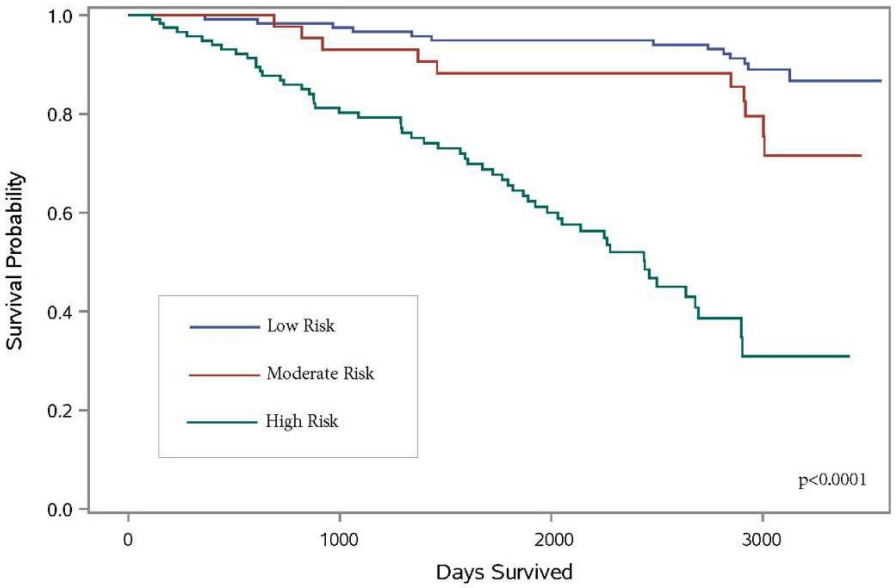

Figure 3. Kaplan Meier survival curves for 10-year cardiovascular mortality stratified by each risk cluster. The following scores were used to derive the risk clusters: SHFM (Seattle Heart Failure Model), FRS (Framingham Risk Score), MAGGIC (Meta-analysis Global Group in Chronic Heart Failure), BCN Bio-HF (Barcelona Bio-Heart Failure Risk Calculator), and Cardiac Lipid Panel Risk Score (CLP). Each prognostic score was standardized to the same scale (mean = 0; SD = 1). Total subjects, n = 280; total events, n = 95.

Study limitations

These proof-of-concept findings should be interpreted as hypothesis generating to be used as a reference for validation studies on larger cohorts in the future. The homogeneity of this cohort, elderly patients with stable CHF, may have had an impact on the performance of the prognostic scores. Due to the inclusion and exclusion criteria of the CIBIS-ELD trial, these results may not have good external validity, and more research would be needed to validate the results. Performance and comparison of the risk scores may be affected by the fact that the models were designed using different endpoint definitions and cohorts. Risk categories that are clinically relevant for one model's definition may not apply to a different model. The MAGGIC score estimates risk of all-cause mortality at one and at three years, the SHFM up to five years, and the BCN Bio-HF at one, two, and three years, and the FRS estimates risk of first CVD event, none of which were developed for the primary outcome of this study of 10-year cardiovascular mortality. The SHFM score may have been affected by the imputation of lymphocytes % as well as the lack of patients taking allopurinol. The BCN-bio HF score was updated in 2018[55] which could provide better predictive value than the 2014 version used in this study. We were limited by the availability of the data for the 2018 version of the BCN-bio HF score, since it required more parameters such as ARNi medication and number of HF hospitalizations in the previous year. The FRS was originally developed for coronary artery disease and not CHF, which may explain its poor performance on this cohort. The CLP biomarker kit was developed for routine use in the clinic; however, it is still a research biomarker panel pending regulatory approval and must be sent to a lab equipped with MS technology. Our findings are limited to this population of elderly CHF patients and future validation studies should be performed to include a more heterogenous cohort such as younger, more women, and early/ asymptomatic patients. Other common biomarkers such as ST2, hs-CRP, and troponins should be compared to the CLP as they are more readily available and do not require samples be sent to a specialized lab. The CLP panel was originally developed as a diagnostic and early detection biomarker for HFrEF, and clinicians and researchers should be cautious when using it as a prognostic tool, as these are still preliminary findings.

Conclusion

In a cohort of ambulatory CHF patients, we have shown that the prognostic scores included in this study were useful in stratifying patients into risk clusters. Our findings demonstrate that the CLP risk score comprising a panel of 3 novel metabolomic biomarkers and NT-proBNP, could improve the prediction of cardiovascular mortality over traditional prognostic scores. In the future, a broader array of biomarkers should be integrated into a more comprehensive risk score that may improve discrimination potential and risk stratification and the

Characteristic	Cluster 1 Low risk n = 119	Cluster 2 Moderate risk n = 44	Cluster 3 High risk n = 117	*p* value
Age (years), mean ± SD	71 ± 5.1	71 ± 4.2	73 ± 4.8	0.0058[a]
NYHA (II/III), n	83/36	35/9	71/46	0.1868[c]
Male, n (%)	91 (77)	29 (66)	86 (74)	0.3979[b]
Body mass index (kg/m^2), mean ± SD	27.0 ± 3.0	27.0 ± 3.4	26.6 ± 3.9	0.4165[a]
Heart rate (bpm), mean ± SD	73.8 ± 26.7	72.0 ± 10.9	73.2 ± 12.6	0.9385[a]
Systolic blood pressure (mm Hg), mean ± SD	136 ± 17.7	137 ± 25.7	131 ± 16.9	0.0642[a]
Diastolic blood pressure (mm Hg), mean ± SD	82.9 ± 10.9	78.7 ± 13.0	79.5 ± 10.3	0.0485[a]
Years since first diagnosis of CHF	5.3 ± 5.9	5.0 ± 4.5	5.3 ± 5.7	0.9737[a]
Cardiac Death, n (%)	24 (20)	12 (27)	59 (50)	< 0.0001[b]
Laboratory, mean ± SD				
Serum creatinine (µmol/l)	103 ± 26.7	104 ± 26.7	113 ± 28.7	0.0034[a]
Hemoglobin (g/dL)	13.6 ± 1.4	13.4 ± 1.1	13.4 ± 1.7	0.1927[a]
Sodium (mmol/L)	142 ± 3.2	141 ± 3.6	141 ± 3.2	0.6576[a]
Uric acid (µmol/L)	342 ± 103	324 ± 108	378 ± 147	0.1084[a]
Total Cholesterol (mmol/L)	5.1 ± 1.6	5.4 ± 1.4	5.0 ± 1.6	0.2022[a]
HDL cholesterol (mmol/L)	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.5	0.6042[a]
LDL cholesterol (mmol/L)	3.5 ± 1.4	3.5 ± 1.1	3.3 ± 1.3	0.4337[a]
Triglycerides (mmol/L)	1.7 ± 0.9	1.7 ± 0.9	1.8 ± 1.0	0.5816[a]
Lymphocytes (%)*	31	31	31	N/A
NT-proBNP (pg/mL)	506.0 (236–1461)[†]	860 (369–1883)[†]	1094 (450–2059)[†]	0.0015[a]
PC 16:0/18:2 (µg/dl)	36,830 (33,035–40,460)[†]	35,300 (30,538–39,370) [†]	37,275 (31,688–39,850) [†]	0.3678 [a]
TAG 18:1/18:0/18:0 (µg/dl)	131 (91–253)[†]	107 (74–264) [†]	103 (72–253) [†]	0.5739 [a]
SM d18:1/23:1, SM d18:2/23:0, SM d17:1/24:1 (µg/dl)	1433 (1181–1613)[†]	1378 (1156–1688) [†]	1296 (1071–1529) [†]	0.1598[a]
Cardiac imaging, mean ± SD				
LVEF (%)	37.7 ± 9.6	36.7 ± 8.2	34.0 ± 9.5	0.0046[a]
LVDed (mm)	57.8 ± 9.2	59.6 ± 8.1	59.5 ± 9.5	0.2684[a]
LVDes (mm)	44.3 ± 9.4	45.6 ± 9.0	46.7 ± 10.1	0.1422[a]
LVVed (mL)	147 ± 57.5	165 ± 77	154 ± 64.6	0.4697[a]
LVVes (mL)	95.9 ± 47	109 ± 60.7	104 ± 52.2	0.4449[a]
LAes (mm)	44.6 ± 6.9	45.3 ± 6.5	45.9 ± 7.7	0.3705[a]
E/e'	11.3 ± 8.8	10.4 ± 9.1	13.0 ± 9.5	0.0202[a]
E/A	1.0 ± 0.6	1.0 ± 0.8	1.2 ± 0.9	0.2407[a]
Deceleration time (ms)	233 ± 84.8	229 ± 80.8	219 ± 72.8	0.4823[a]
Comorbidities, n (%)				
Diabetes	29 (24)	11 (25)	42 (36)	0.1195[b]
Hypertension	95 (80)	39 (87)	90 (77)	0.2534[b]
Coronary artery disease	80 (67)	30 (68)	90 (77)	0.2245[b]
Smokers	55 (46)	20 (46)	50 (43)	0.8592[b]
Hyperlipidemia	67(56)	26 (61)	69 (59)	0.8636[b]
COPD	2 (2)	2 (5)	5 (4)	0.2143[b]
Medication, n (%)				
ACE inhibitor	104 (87)	36 (82)	107 (92)	0.2244[b]
Allopurinol	0 (0)	0 (0)	0 (0)	N/A
ARB	39 (33)	16 (36)	60 (51)	0.0121[b]
Beta blocker	87 (73)	36 (82)	80 (68)	0.2303[b]
Diuretics	53 (45)	23 (52)	69 (59)	0.0850[b]
Diuretic dose mg/kg, mean ± SD	0.28 ± 0.25	0.33 ± 0.35	0.36 ± 0.34	0.3410[a]
Glycoside	26 (22)	8 (18)	25 (21)	0.8736[b]
Aspirin	92 (77)	33 (75)	91 (78)	0.9309[b]
Nitrate	55 (46)	28 (64)	63 (54)	0.1262[b]
Antiarrhythmic agent	16 (13)	6 (14)	20 (17)	0.7075[b]
Statin	51 (43)	18 (41)	45 (39)	0.7894[b]
Continued				

Characteristic	Cluster 1 Low risk n = 119	Cluster 2 Moderate risk n = 44	Cluster 3 High risk n = 117	*p* value
Prognostic scores				
SHFM, Mean (SD)	1.1 ± 0.54	0.9 ± 0.6	1.32 ± 0.5	< .0001[a]
FRS, Mean (SD)	20.1 ± 4.1	20.6 ± 4.5	20.1 ± 4.0	0.6033[a]
MAGGIC, Mean (SD)	22.1 ± 5.0	21.4 ± 3.8	22.7 ± 5.0	0.2092[a]
BCN Bio-HF, Mean (SD)	0.83 ± 0.17	0.34 ± 0.13	0.76 ± 0.2	< .0001[a]
CLP, n (%)				< .0001[b]
0	6 (6)	1 (3)	0 (0)	
1	83 (85)	24 (75)	4 (4)	
2	4 (4)	3 (9)	13 (12)	
3	5 (5)	4 (13)	77 (68)	
4	0 (0)	0 (0)	19 (17)	

Table 3. Comparison of cohort characteristics and prognostic scores. Cohort characteristics and prognostic score distribution across risk clusters. The prognostic scores used for clustering were: SHFM (Seattle Heart Failure Model), FRS (Framingham Risk Score), and MAGGIC (Meta-analysis Global Group in Chronic Heart Failure), BCN Bio-HF (Barcelona Bio-Heart Failure Risk Calculator), and Cardiac Lipid Panel Risk Score (CLP). Each prognostic score was standardized to the same scale (mean = 0; SD = 1). Ward's minimum variance method was used for clustering. ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; TAG, triacylglycerol 18:1/18:0/18:0; COPD, chronic obstructive pulmonary disease; E/A, ratio of the early (E) to late (A) ventricular filling velocities; E/e', ratio between early mitral inflow velocity and mitral annular early diastolic velocity; LAes, left atrial end systole; LDL, low-density lipoprotein; NYHA, New York Heart Association; HDL, high-density lipoprotein; LVDed, left ventricular diameter end diastole; LVDes, left ventricular diameter end systole; LVVed, left ventricular volume end diastole; LVEF, left ventricular ejection fraction; LVVes, left ventricular volume end systole; mg/kg, milligrams per kilograms; NTpro-BNP, N-terminal pro–B-type natriuretic; PC, phosphatidylcholine; SM, sphingomyelin. [a]Wilcoxon rank sum test, [b]Pearson's chi-square test, [c]Mantel-Haenszel chi-square. *Imputed using the median, 31%, of the normal range 20–40%. [†]Median (Interquartile Range).

CLP offers a promise. The CLP score is a step in the direction of providing a more precise decision support tool to assist clinicians and patients in managing their CHF treatment.

Methods

Study population. This study used a sub-cohort randomly selected from the Cardiac Insufficiency Bisoprolol Study in Elderly (CIBIS-ELD) trial, a multi-center, randomized, double-blind trial with ≥ 65-year-old patients being treated for CHF. The original study design and results of the CIBIS-ELD trial have been published previously[56,57]. Briefly, patients with CHF were randomized in a 1:1 fashion to receive two different beta-blockers, either bisoprolol or carvedilol, and up titrated every fortnight for 12 weeks and then followed at 10 years. From this source cohort (n = 883), there were n = 589 with available blood samples. Patients were randomly selected and included in the analysis only if they passed quality control[58,59] resulting in a final set of 280 cases. The ethics committees of all participating centers approved the study protocol, and informed consent was signed by all participants prior to study participation. The ethics committees include: Germany: Ethikkommission der Charité on the 13th June 2007 (Amendment 5) (ref: 125/2004), Serbia: Ethics board of the University Hospital on the 31st March 2006 (ref: 6108/18), Slovenia: The national medical ethics committee on the 2nd July 2007 (ref: KME 188/06/07). The investigation conformed to the principles outlined in the Declaration of Helsinki[60].

Biomarker measurements. Targeted metabolite profiling of the serum samples which passed quality control was performed at a specialized metabolomics lab using a commercially available kit. The kit uses a protocol based on a 1-phase extraction of the blood samples followed by gas chromatography mass spectrometry (GC–MS) (Agilent 6890 GC coupled to an Agilent 5973 MS-System) and liquid chromatography tandem-mass spectrometry (LC–MS/MS) (Agilent 1100 HPLC-System coupled to an Applied Biosystems API4000 MS/MS-System) analysis as previously described[29]. The analytical protocol was designed for routine measurement in the clinical practice setting; however, it is currently only available in specialized labs equipped with MS technology. The samples were stored at − 80 °C and transferred on dry ice prior to analysis. The three CLP metabolomic features and NT-proBNP measurements, were generated at baseline, only for the previously mentioned samples (n = 280). NT-proBNP was a measured using commercially available assays (Elecsys, Roche Diagnostics).

Calculating prognostic scores. Each prognostic score was calculated using the corresponding method proposed by the original authors (3–6). Only the scores which were developed in the follow-up time period, 2006–2016, were included in the analysis due to data availability. For calculating the SHFM score, % lymphocyte was missing, and the median (31%) of the normal range (20–40%) was imputed for all subjects. For calculating

the BCN Bio-HF score, the model with clinical variables plus NT-proBNP was used since ST-2 and hs-cTnT were not available. The CLP risk score was calculated as the count of biomarkers above the Youden index cut-off[61]. The Youden's index calculates each biomarker's optimal cut-off from the Cox regression. There were 4 cut-off values, since four biomarkers are included in the score: three from the CLP and NT-proBNP. Based on the cut-off, a value of 1 or 0 was assigned if the biomarker value was above/below the cut-off value, or in the direction of greater risk, then all 4 values were summed to generate the final score for each subject. The score ranged from 0 to 4, higher scores indicating higher risk. The primary outcome, cardiovascular death, was defined as death by myocardial infarction, non-responding arrhythmia, asystole, chronic pump failure, or other cardiac cause and verified by a blinded committee of cardiologists.

Statistical analysis. *Power and sample Size.* The sample size was adjusted for an anticipated event rate of 0.34. A Cox regression of the log hazard ratio on a covariate with a standard deviation of 1.5 based on a sample of 257 observations achieves 80% power at a 0.050 significance level to detect a regression coefficient equal to 0.2. Adjusting for an anticipated loss to follow up rate of 10%, the final sample size would be 283.

Discrimination analysis and calibration. Categorical variables were expressed as number (%) and continuous variables were expressed as mean (*SD*). The primary outcome was 10-year cardiovascular death, and the secondary outcome was 3 year all-cause death, since all scores except for FRS were developed for this outcome. Cox Regression was performed on each of the prognostic scores, and hazard ratios and 95% confidence intervals were calculated to assess their relationship with the outcome.

For the survival models, integrated area under the receiver operator curves (IAUC) and Harrell's c statistic[62] were calculated to assess the discrimination of each score in predicting the outcome. Hypothesis testing of the change in discrimination was performed by calculating the differences in concordance statistics[63]. The IAUC curves are computed as a weighted average of the AUC values at all the event times, with the weights as the jumps of the Kaplan–Meier estimate of the survivor function. Calibration (i.e., the agreement between observed outcomes and predictions) of all models was assessed graphically, with calibration plots.

Competing event and cause-specific analysis was performed for all models with non-cardiovascular mortality as the competing event. The cumulative incidence function (CIF) was calculated for the CLP which was stratified by low (CLP score 0–1), moderate (CLP score 2), and high (CLP score 3–4) to assess CIF. The discrimination analysis and competing event and cause-specific analysis were performed using SAS software version 9.4 of the SAS System for Windows (SAS Institute, Inc., Cary, North Carolina)[64]. Calibration was analyzed using Stata Statistical Software version 16[65].

Cluster analysis. Hierarchical cluster analysis was performed using Ward's minimum variance method to assess each prognostic score's ability to separate cases into risk groups. The distance between two clusters is the ANOVA sum of squares between the clusters summed over all variables. Only the 5 risk scores used as the input variables for the cluster analysis to examine how well they classified patients into a low, moderate, and high-risk of cardiovascular mortality. Data was standardized (mean of 0 and SD of 1), to perform clustering. The clinical characteristics and scores were compared across risk clusters. Comparisons among continuous variables were performed using Wilcoxon rank sum test; and Pearson's chi-square test (or Fisher's exact test) or Mantel–Haenszel Chi-square test for categorical and ordinal data, respectively. Kaplan–Meier curves were used to compare the survival distribution across risk clusters. Survival time was calculated from baseline until cardiovascular death or censoring at 10 year follow up. Cluster analysis was performed using JMP pro software version 14[66]. Kaplan–Meier curves were generated using SAS software version 9.4 of the SAS System for Windows (SAS Institute, Inc., Cary, North Carolina)[64].

Correlation analysis. To investigate potential relationships between the CLP biomarker values and common clinical parameters, Pearson's correlation coefficients were calculated, significant at the 0.01 level (2-tailed). Correlation analysis was performed using R software version 3.6.1[67].

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Author contributions
Author T.T. was responsible for study conception and design; authors B.P., E.T., F.E., G.L., H.D.D., and T.T. were responsible for acquisition of data; authors A.S., D.O., E.V., F.B., J.S., M.R., P.L., P.M., and S.A. and were responsible for data analysis, and drafting and revision of the manuscript. All authors critically reviewed and approved the manuscript.

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Competing interests
The authors declare no competing interests.

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11. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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12. Publication List

12.1 Articles

12.1.1 First Authorship:

1. **McGranaghan P**, Saxena A, Rubens M, Radenkovic J, Bach D, Schleußner L, Pieske B, Edelmann F, Trippel TD. Predictive value of metabolomic biomarkers for cardiovascular disease risk: a systematic review and meta-analysis. Biomarkers. 2020 Feb 17;25(2):101-11.
   Impact factor 2019: 2.070

2. **McGranaghan P**, Düngen HD, Saxena A, Rubens M, Salami J, Radenkovic J, Bach D, Apostolovic S, Loncar G, Zdravkovic M, Tahirovic E, Veskovic J, Störk S, Veledar, E, Pieske B, Edlemann F, Trippel T. Incremental prognostic value of a novel metabolite-based biomarker score in congestive heart failure patients. ESC heart failure. 2020 Oct;7(5):3029-39.
   Impact factor 2019: 3.902

3. **McGranaghan P**, Saxena A, Düngen HD, Rubens M, Appunni S, Salami J, Veledar E, Lacour P, Blaschke F, Obradovic D, Loncar G, Apostolovic E, Edelmann F, Pieske B, Trippel T. Performance of a cardiac lipid panel compared to four prognostic scores in chronic heart failure. Scientific Reports. 2021 Apr 14;11(1):1-2.
   Impact factor 2019: 3.998

12.1.2 Co-Authorship:

4. Rubens M, Ramamoorthy V, Saxena A, Das S, Bhatt C, Veledar E, **McGranaghan P**, Sundil S, Shehadeh N, Viamonte-Ros A, Linhares Y, Odia Y, Chuong M, Rupesh K, Mehta MP. Recent Health Care Expenditure Trends Among Adult Cancer Survivors in United States, 2009-2016. American journal of clinical oncology. 2020 May 14;43(5):349-55.
   Impact Factor 2019: 1.907

5. Rubens M, Ramamoorthy V, Saxena A, Bhatt C, Das S, Veledar E, **McGranaghan P**, Viamonte-Ros A, Odia Y, Chuong M, Kotecha R. A risk model for prediction of 30-day readmission rates after surgical treatment for colon cancer. International journal of colorectal disease. 2020 Aug;35:1529-35.
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Shehadeh N, Veledar E, Viamonte-Ros A, Odia Y, Mehta MP, Kotecha R. Inpatient Palliative Care Use Among Critically Ill Brain Metastasis Patients in the United States. American journal of clinical oncology. 2020 Nov 4;43(11):806-12.
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8. Appunni S, Rubens M, Ramamoorthy V, Anand V, Khandelwal M, Saxena A, **McGranaghan P**, Odia Y, Kotecha R, Sharma A. Lumican, Pro-tumorigenic or Anti-tumorigenic: A Conundrum. Clinica Chimica Acta. 2020 Dec 15. *Impact Factor 2019: 2.615*

9. Ramamoorthy V, Rubens M, Saxena A, Bhatt C, Das S, Appunni S, Veledar E, **McGranaghan P**, Shehadeh N, Viamonte-Ros A, Linhares Y, Odia Y, Kotecha R, Mehta MP. Prevalence and Inpatient Hospital Outcomes of Malignancy-Related Ascites in the United States. American Journal of Hospice and Palliative Medicine®. 2021 Jan;38(1):47-53. *Impact Factor 2019: 1.638*

10. Saxena A, Rubens M, Ramamoorthy V, Tonse R, Veledar E, **McGranaghan P,** Sundil S, Chuong MD, Hall MD, Odia Y, Mehta MP, Kotecha R. Hospitalization rates for complications due to systemic therapy in the United States. Scientific reports. 2021 Apr 1;11(1):1-7. *Impact factor 2019: 3.998*

12.2 Abstracts and Posters

11. **McGranaghan P**, Saxena A, Rubens M, Radenkovic J, Bach D, Schleußner L, Pieske B, Edelmann F, Trippel T. Abstract P412: Toward A Biomarker Kit Suitable For Clinical Practice; A Novel Metabolomic Biomarker Risk Score Improves The Prognostic Value Of Cardiovascular Mortality In Elderly Congestive Heart Failure Patients. Circulation. 2020 Mar 3;141(Suppl_1):AP412-.

12. **McGranaghan P,** Saxena A, Rubens M, Garelnabi MO, Veledar E, Trippel T. Abstract MP139: Role of a Novel Cardiovascular Lipid-based Biomarker Score in Identifying Candidates for Beta Blocker Intervention. Arteriosclerosis, Thrombosis, and Vascular Biology. 2020 May;40(Suppl_1):AMP139-.

13. Saxena A, Rubens M, Ramamoorthy V, Das S, Bhatt CB, Salami JA, **McGranaghan P**, Shehadeh N, Veledar E. Abstract P338: Cardiovascular And Cerebrovascular Events Associated With Cancer Surgery In The United States. Circulation. 2020 Mar 3;141(Suppl_1):AP338-.

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15. Saxena A, Rubens M, Salami JA, **McGranaghan P**, Bhatt CB, Das S, Ramamoorthy V, Veledar E. Abstract P340: 30-Day Readmission Risk Assessment Among Patients Who Underwent Transcatheter Aortic Valve Replacement (TAVR), ACS NSQIP, 2015-2017. Circulation. 2020 Mar 3;141(Suppl_1):AP340-.

16. Saxena A, **McGranaghan P**, Das S, Rubens M, Salami J, Veledar E. Abstract WP242: Modeling Prevention, Prediction or Explanation of Stroke Risk: Insights From Meta-Analysis of Studies With Models Predicting Stroke or Composite Outcomes. Stroke. 2019 Feb;50(Suppl_1):AWP242-.

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