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RESEARCH ARTICLE

Proteomic prediction of incident heart failure and its main subtypes

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Aim To examine the ability of serum proteins in predicting future heart failure (HF) events, including HF with reduced or preserved ejection fraction (HFrEF or HFpEF), in relation to event time, and with or without considering established HF-associated clinical variables.

Methods and results In the prospective population-based Age, Gene/Environment Susceptibility Reykjavik Study (AGES-RS), 440 individuals developed HF after their first visit with a median follow-up of 5.45 years. Among them, 167 were diagnosed with HFrEF and 188 with HFpEF. A least absolute shrinkage and selection operator regression model with non-parametric bootstrap were used to select predictors from an analysis of 4782 serum proteins, and several pre-established clinical parameters linked to HF. A subset of 8–10 distinct or overlapping serum proteins predicted different future HF outcomes, and C-statistics were used to assess discrimination, revealing proteins combined with a C-index of 0.80 for all incident HF, 0.78 and 0.80 for incident HFpEF or HFrEF, respectively. In the AGES-RS, protein panels alone encompassed the risk contained in the clinical information and improved the performance characteristics of prediction models based on N-terminal pro-B-type natriuretic peptide and clinical risk factors. Finally, the protein predictors performed particularly well close to the time of an HF event, an outcome that was replicated in the Cardiovascular Health Study.

Conclusion A small number of circulating proteins accurately predicted future HF in the AGES-RS cohort of older adults, and they alone encompass the risk information found in a collection of clinical data. Incident HF events were predicted up to 8 years, with predictor performance significantly improving for events occurring less than 1 year ahead, a finding replicated in an external cohort study.

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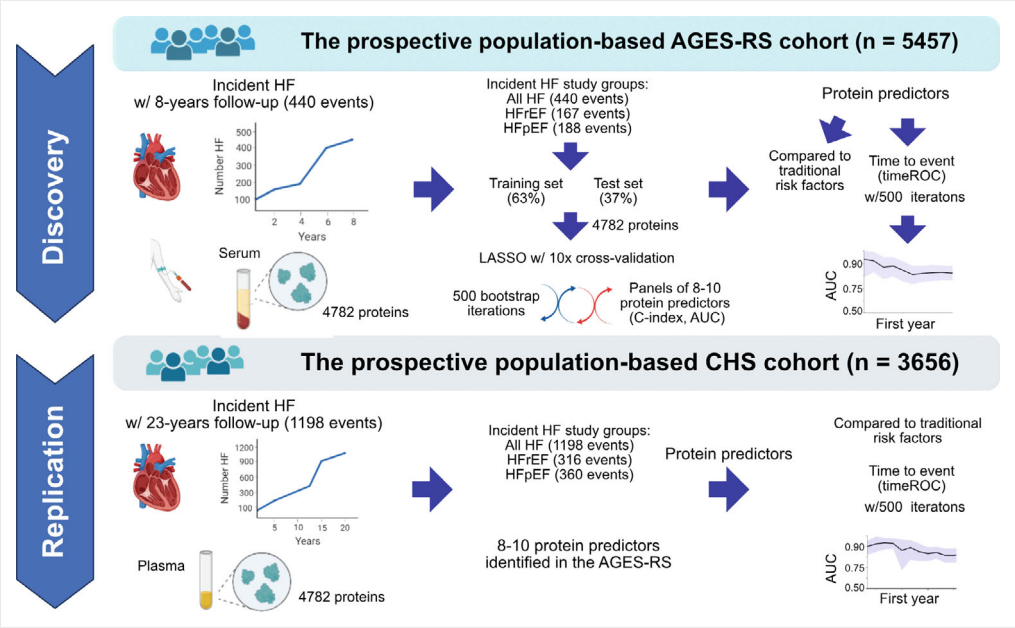
Graphical Abstract



The ability of the deep circulating proteome to predict future heart failure (HF) events, including its primary subtypes, in relation to event time and known HF-associated clinical factors was studied in two prospective population-based cohorts. AGES-RS, Age, Gene/Environment Susceptibility Reykjavik Study; CHS, Cardiovascular Health Study; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristic.

Keywords Heart failure • Serum proteomics • Clinical variables • C-statistics • Predictors • Event time

Introduction

Heart failure (HF) is not a single medical diagnosis, but a clinical syndrome characterized by cardinal symptoms that are frequently accompanied by signs and is caused by a structural and/or functional heart defect.[1] This can lead to elevated intracardiac pressures and/or insufficient cardiac output at rest and/or during exercise.[1] Over the last few decades, various medical interventions have improved the survival of HF patients.[2–5] However, the mortality remains high and the exact percentages are influenced by the underlying aetiology, comorbidities, stage of the disease (acute vs. chronic HF), study population and diagnostic criteria.[6] HF is estimated to have a population prevalence of 2%, which is steadily increasing as the population ages.[7] Approximately equal numbers of newly hospitalized individuals with HF have HF with reduced ejection fraction (HFrEF) or HF with preserved ejection fraction (HFpEF).[7]

There are many underlying aetiologic and pathophysiologic factors that influence the risk of HF. Recent epidemiological research indicates that the incidence of HFpEF is growing faster than that of HFrEF,[8] which may be explained by rapid increases in HFpEF-associated risk variables such as type 2 diabetes (T2D) and obesity.[9–11] Other risk factors such as history of myocardial infarction (MI), uncontrolled hypertension and valve failure are linked to HFrEF.[10,12] Endothelial dysfunction is also more noticeable in HFpEF than in HFrEF, and patients with HFpEF are more likely to be women.[10] In contrast, cardiomyocyte loss, fibrosis, and cardiomyocyte hypertrophy are all common features of HFrEF.[10] HF is therefore a heterogeneous disorder where the epidemiology and pathophysiology of the two major subtypes differ markedly.

Accurate prediction of new-onset HF allowing for the identification of high-risk individuals, offers early focused intervention and hence improved patient outcomes. Deep serum proteomics has revealed links between circulating proteins and diseases of various aetiologies,[13–19] with recent discoveries fueled by aptamer-based affinity methods in particular.[13,16,20–22] In the current study, levels of 4782 serum proteins encoded by 4137 distinct genes, including the most reliable clinical diagnostic biomarker for HF, N-terminal pro-B-type natriuretic peptide (NT-proBNP),[1] measured in the prospective population-based Age, Gene/Environment Susceptibility Reykjavik Study (AGES-RS) cohort, were examined individually and in the context of multiple clinical variables for prediction of future HF events. The AGES-RS findings were followed up on in the Cardiovascular Health Study (CHS).

Methods

Study population

Cohort participants aged 66 through 96 years at the time of blood collection were from the AGES-RS,[23] a single-centre, prospective, population-based study of older adults ($n = 5764$, mean age 76.6 ± 6 years). The AGES-RS was formed between 2002 and 2006, and its participants were randomly selected from the surviving members of the established 40-year-long population-based prospective Reykjavik study,[24,25] with a 72% recruiting rate. The Reykjavik study, a prospective cardiovascular survey, recruited a random sample of 30 795 adults born between 1907 and 1935 who lived in the greater Reykjavik area in 1967, that were examined in six phases from 1967 to 1996.[24,25] Furthermore, the Reykjavik study accurately represents the entire country because 65.3% of participants came from all around Iceland, resulting in a proportional representation of the overall population at the time of birth. The AGES-RS measurements, which include for instance brain and vascular imaging, are designed to assess four biologic systems: vascular, neurocognitive (including sensory), musculoskeletal, and body composition/metabolism.[23] All AGES-RS participants are of European ancestry. A decade-long collaboration with large genetic and epidemiology consortia of multiple disease-related phenotypes revealed no discernible difference between the Icelandic population and other European ancestry cohorts.[26–28] AGES-RS was approved by the National Bioethics Committee in Iceland that acts as the institutional review board for the Icelandic Heart Association (approval number VSN-00-063, in accordance with the Helsinki Declaration) and by the US National Institutes of Health, National Institute on Aging Intramural Institutional Review Board.

Prevalent coronary heart disease (CHD) was defined as previous or prevalent MI, coronary artery bypass graft or percutaneous coronary intervention obtained from hospital records at AGES-RS visit. Incident CHD events included fatal CHD or incident non-fatal CHD (International Classification of Diseases [ICD] 9th edition, codes 410, 411, 414, 429, and ICD 10th edition, codes I21-I25), obtained from cause of death registries and hospitalization records from the National University Hospital, the main provider of tertiary care in Iceland. Systolic and diastolic blood pressure were measured twice with subjects in a supine position using a mercury sphygmomanometer. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared, lipoproteins and plasma glucose levels were measured on fasting blood samples. Triglyceride was measured using enzymatic colorimetry (Roche Triglyceride Assay Kit), high-density lipoprotein (HDL) cholesterol with an enzymatic *in vitro* assay (Roche Direct HDL Cholesterol Assay Kit), and glucose was measured using photometry (Roche Hitachi 717 Photometric Analysis System). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation. T2D was determined from self-reported diabetes, diabetes medication use, or fasting plasma glucose ≥7 mmol/L according to the American Diabetes Association guidelines.[29]

Coronary artery calcium (CAC) was quantified using the Agatston scoring method,[30] which was reviewed independently by four image analysts. Phantom-adjusted CAC was expressed as a sum score for all four coronary arteries, as previously described in greater detail.[31] Thoracic aorta calcium (TAC) was quantified using the Agatston method.[30] TAC was scored in the proximal descending thoracic aorta (from the inferior border of the transverse arch to the level of the aortic bulb), and the distal descending thoracic aorta (from the level of the aortic bulb to the bottom of the left ventricular apex). The calcium score of each lesion was calculated by multiplying the lesion area by a density factor derived from the maximum Hounsfield units (HU) within this area.

The criteria for HF were based on clinical symptoms and signs, chest X-ray, and, in many cases echocardiographic findings from hospital records, which were adjudicated by examining every record for both prevalent HF, that is had HF at the baseline visit, and incident, that is HF diagnosed after the baseline visit. The incident HF cases were participants of the AGES-RS who were free of HF diagnosis at the baseline visit, but who were later hospitalized and diagnosed (hospital discharge ICD-10 diagnosis codes starting with I50) with HF during the follow-up period of 8 years. Each patient's thorough medical records were subsequently adjudicated by a cardiologist in accordance with the pre-specified criteria of AGES-RS,[23] to confirm the diagnosis of symptomatic HF and the date of the incident HF event documented. Among the criteria were symptoms such as shortness of breath that could be ambulatory, and signs of pulmonary oedema. Further, information on cardiac ultrasound (i.e. echocardiography) to determine left ventricular ejection fraction (LVEF) and comorbid conditions were also collected for each patient. Echocardiographic measures of LVEF were used to classify HF subgroups. Individuals with HFpEF had a LVEF of ≥50% whereas those with HFrEF had and LVEF of ≤40%.

Serum protein measurements

Blood samples were collected at the AGES-RS baseline visit after an overnight fast, and serum prepared using a standardized protocol and stored in 0.5 ml aliquots at −80°C. Serum samples collected from the inception period of AGES-RS, that is, from 2002 to 2006, were utilized to generate proteomics data. Before the protein measurements, all serum samples from this period went through their first freeze−thaw cycle. Serum protein levels were determined using a multiplex SOMAscan proteomic profiling platform (Novartis V3-5K) which employs SOMAmers (Slow-Off rate Modified Aptamers) that bind to target proteins with high affinity and specificity.[16] The custom-design SOMAscan platform was built to quantify proteins that are known or expected to be present extracellularly or on the surface of cells.[16] The proteomics platform-specific aptamers which target 4782 human proteins, map to 4137 gene identities where some proteins were targeted by more than one aptamer. In such cases, individual aptamers had distinct binding sites (epitopes) or binding affinity.[16] The single gene *NPPB*, for example, produces three protein products: full-length BNP, NT-proBNP, and BNP32, each of which are targeted by different aptamers. Duplicate aptamers to single pass transmembrane proteins (one to extracellular domain and another to intracellular loop), aptamers targeting multimers (e.g. interleukins), and duplicate aptamers generated in distinct expression systems are further examples. Proteins targeted by multiple aptamers received the same treatment during the analyses as those targeted by a single aptamer. Of the 5764 AGES-RS participants, 5457 were measured for the serum proteome.[16] The aptamer-based proteomics platform measures proteins with femtomole (fM) detection limits and a broad detection range or >8 logs of concentration. To avoid batch or time-of-processing biases, the order of sample collection and separately, sample processing for protein measurements were randomized and all samples run as a single set at SomaLogic Inc. (Boulder, CO, US). The platform exhibits ~5% coefficients of variation for median intra- and interassay variability.[16] SomaLogic performed the assays in collaboration with Novartis, according to the protocol described by our group.[16] Several metrics, including aptamer specificity through direct tandem mass spectrometry analysis and inferential assessment via genetic analysis, have been used to determine the performance of the proteomic platform, suggesting robust target specificity throughout

the platform.[16] Protein data were centred, scaled and Box–Cox transformed,[32] and extreme outlier values excluded, defined as values above the 99.5th percentile of the distribution of 99th percentile cutoffs across all proteins after scaling, resulting in the removal of an average 11 samples per aptamer (~2% of samples/aptamer).

Statistical analysis

The least absolute shrinkage and selection operator (LASSO) model,[33,34] and non-parametric bootstrap,[35] were used to approximate the sampling distribution of proteomic variables' coefficients in age- and sex-adjusted logistic and Cox proportional hazards regression models as well as their predictive performance as measured by the area under the curve (AUC) and the Harrel's concordance index (C-index).[36] This allows us to assert ambiguity in model parameters and instability.[37,38] Harrel's C-index is best known for its intuitive and straightforward interpretation, as it evaluates the predictor's ability to order events by estimating the fraction of correctly ordered pairs out of all comparable pairs in the dataset. In brief, we created two datasets that were repeated for each bootstrap sample. For the training dataset, the bootstrap selects datapoints from approximately 63% of the samples. These were used to fit the LASSO models and estimate the coefficients. For the testing dataset, the datapoints not selected by the bootstrap and comprising ~37% of the data were used to estimate the model's out-of-sample predictive performance, expressed as AUC and C-index. Following that, we took the training dataset and performed 10-fold cross-validation by dividing the samples into 10 equally sized groups and selecting one of the 10 groups to exclude from the model fitting process. The LASSO path was then fitted to the remaining nine groups using a set of values for the regularization coefficent's lambda. The prediction error for the left-out tenth group was calculated for each value of lambda. The prediction errors were averaged for each value of lambda, and each of the 10 models fitted in this manner provided an error curve. We used this to determine which value of lambda minimizes the error curve and the LASSO model re-fitted on the entire training dataset, and to return the coefficient values from that model. Finally, the coefficients calculated on the training dataset were used to compute out-of-sample prediction errors on the testing dataset. This procedure was carried out 500 times.

Based on the boLASSO,[39] and stability selection methods,[40] a protein was classified as important to the prediction of HF if it had an estimated non-zero coefficient in at least 80% of 500 bootstrap replications. After determining which proteins were important for prediction, we conducted additional analyses to decide whether including these proteins in clinical prediction models would improve prediction quality. This was done for both individual proteins and all proteins selected by LASSO-bootstrap iterations, combined. Here each bootstrap iteration gives us the estimated coefficients for proteins. For the combined protein panel, we used these coefficients to compute a weighted sum of the measured protein values, yielding a single number for each participant that is the log of the hazard ratio for that participant, which we can use to compare the relative hazards between participants. We used the non-parametric bootstrap to approximate the out-of-sample concordance of the models by sequentially adding variables to the models and considering which ones added to the predictive capability by comparing the performance of each model on the same bootstrap samples and calculating 95% confidence intervals on the pairwise differences in AUC and C-index. In all analyses we adjusted for age and sex, and for LASSO models the coefficients for age and sex were unpenalized.

The timeROC R package[41] was used to perform inverse probability of censoring weighting (IPCW) estimation of cumulative/dynamic time-dependent receiver operating characteristic (ROC) curves, which was then used to calculate the time-dependent AUC, monthly over a 8-year period, for the different protein predictor panels. The time to event estimates for the various protein predictors did not include demographic or clinical variables. To obtain an estimate of the uncertainty for the protein predictor panels we performed 500 bootstrap replications of the time-dependent ROC curves and calculated the mean, 2.5% quantile, and 97.5% quantile at each time point. All analyses of the current study were conducted using R version 4.2.1. and R Rstudio (1.1.456).

Replication cohort

Replication of the AGES-RS analyses was performed in the CHS, a population-based U.S. cohort study of risk factors for cardiovascular disease in adults aged 65 and older.[42] Participants in the CHS were recruited in 1989–1990 ($n = 5201$) and again in 1992–1993 ($n = 687$), when the African-American cohort was recruited. A subset of CHS participants with available plasma specimens at the 1992–1993 exam underwent proteomics measurement with the SomaScan 5K platform.[43] The CHS cohort was evaluated semi-annually for ascertainment and central adjudication of incident HF events over long-term follow-up.[44,45] HF events were ascertained by self-report and by screening the diagnostic codes of all hospitalizations.[45,46] The Cardiovascular Events Committee reviewed all events, and an HF event required symptoms, a physician diagnosis, plus evidence from the physical exam, diagnostic tests such as chest X-rays and echocardiograms, and the initiation of some form of treatment.[45,46] After excluding prevalent HF at the 1992–1993 baseline, there were 3484 participants with available proteomics measures who experienced first-ever 1198 HF events (316 HFrEF and 360 HFpEF) over 23 years of follow-up. The protein predictions scores were recalculated by fitting the model weights determined in the AGES-RS discovery dataset in CHS again using bootstrap resampling. The CHS proteomics data were log2 transformed and standardized to have a mean of zero and standard deviation of one. The methodologies, processes, and proteome assessment in the CHS are detailed in online supplementary material.

Results

Incidence of heart failure and baseline characteristics of the AGES-RS cohort

In the AGES-RS cohort, 612 individuals were diagnosed with HF, with 440 being diagnosed after blood was drawn at the baseline visit. *Table 1* displays selected measures of the baseline characteristics of the AGES-RS cohort including sex-stratified demographic, biochemical, clinical, physiological, anthropometric, and computed tomography (CT) imaging data on coronary artery and thoracic aorta calcification (CAC and TAC), as well as the prevalence and incidence of HF, and HF-related disease endpoints.

Given the significance of identifying protein predictors for future HF in this study, the characteristics of the incident HF group were of particular relevance. This study excluded all participants who had been diagnosed with HF before to enrolling in the AGES-RS. Thus, the 440 incident HF patients were AGES-RS subjects who were not diagnosed with HF at the baseline visit but were hospitalized and diagnosed with HF throughout the 8-year follow-up period (see *Methods*). The incident cases had a

Table 1 Age, Gene/Environment Susceptibility Reykjavik study cohort baseline characteristics with serum proteome measurements

Characteristic	Male	Female	Total
Demographics			
Numbers	2330 (43)	3127 (57)	5457
Age (years)	76.7 (5.4)	76.5 (5.7)	76.6 (5.6)
Anthropometry			
BMI (kg/m^2)	26.9 (3.8)	27.2 (4.8)	27.1 (4.4)
Obese (BMI ≥30 kg/m^2)	439 (18.9)	777 (24.9)	1216 (22.3)
Lifestyle			
Smoker (current)	265 (11.7)	390 (12.8)	655 (12.3)
Physiological			
DBP (mmHg)	76.2 (9.6)	72.2 (9.5)	73.9 (9.7)
SBP (mmHg)	143.2 (20.4)	142.2 (20.9)	142.6 (20.7)
HbA1c	0.51 (0.1)	0.47 (0.08)	0.49 (0.09)
HDL-C (mmol/L)	1.4 (0.4)	1.7 (0.4)	1.6 (0.4)
LDL-C (mmol/L)	3.2 (1)	3.7 (1)	3.5 (1)
TG (mmol/L)	1 [0.8–1.4]	1.1 [0.8–1.5]	1 [0.8–1.4]
Cardiovascular imaging			
CAC	622.9 [170.7–1513.7]	148.8 [14.8–568.8]	296.4 [47.1–945.5]
TAC	216.5 [26.7–912]	295.6 [38.9–1078.1]	261.3 [31.9–998.9]
Plaque	1493 (69.4)	1891 (66.0)	3384 (67.5)
Metabolic			
T2D	365 (15.7)	293 (9.4)	658 (12.1)
MetS	486 (20.9)	641 (20.5)	1127 (20.7)
Cardiovascular			
CHD, prevalent	777 (33.6)	440 (14.2)	1217 (22.5)
MI, prevalent	427 (18.5)	242 (7.8)	669 (12.4)
HTN	1877 (80.6)	2542 (81.3)	4419 (81)
HF total	334 (14.5)	278 (9)	612 (11.3)
HF total, incident	233 (10.1)	207 (6.7)	440 (8.1)
HF total, prevalent	101 (4.4)	71 (2.3)	172 (3.2)
HFpEF total	103 (4.5)	135 (4.4)	238 (4.4)
HFpEF, incident	81 (3.5)	107 (3.5)	188 (3.5)
HFpEF, prevalent	22 (0.9)	28 (0.9)	50 (0.9)
HFrEF total	159 (6.9)	78 (2.5)	237 (4.4)
HFrEF, incident	112 (4.8)	55 (1.8)	167 (3.1)
HFrEF, prevalent	47 (2.0)	23 (0.7)	70 (1.3)
HF follow-up (years)	5.3 [4.4–6.3]	5.5 [4.8–6.5]	5.4 [4.7–6.4]

Values are given as mean (standard deviation) for continuous variables, *n* (%) for categorical variables, and median [interquartile range] for skewed variables.
BMI, body mass index; CAC, coronary artery calcium; CHD, coronary heart disease; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; HDL-C, high-density lipoprotein HDL cholesterol; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LDL-C, low-density lipoprotein cholesterol; MetS, metabolic syndrome; MI, myocardial infarction; SBP, systolic blood pressure; T2D, type 2 diabetes; TAC, thoracic aorta calcium; Plaque, presence of carotid plaque (carotid plaque was assessed in 5017 individuals of the AGES-RS cohort); TG, triglyceride.

median follow-up time of 5.45 years (range 0.005–7.77 years) and an incidence rate of 1.58 cases per 100 person-years at risk. After adjusting for age at diagnosis, HF patients had a 5-year survival rate of 32.5% and a median survival time of 2.87 years. Of the 440 incident HF patients with echocardiographic data, 167 had HFrEF (LVEF ≤40%) and 188 had HFpEF (LVEF ≥50%) (*Table 1*).

Serum proteins that predict all incident heart failure events

In order to identify independent predictors of all incident HF events, which included all ejection fraction categories as well as those without echocardiographic data, we estimated the sampling distribution of logistic regression coefficients for all 4782 proteins, measured in serum samples from the baseline visit, as well as a number of known HF risk clinical variables using LASSO regression,[33] and non-parametric bootstrap.[35] We applied the LASSO regression to complete 500 iterations of the 10-fold cross-validation step in the AGES-RS discovery cohort to provide internal validation of findings (*Figure 1*), where the bootstrap validated concordance probability estimate (C-index) was based on out-of-sample predictions. The Harrel's C-index was employed to assess the goodness of fit when modelling the prognostic risk scores over the 8 years of follow-up.[36] For serum proteins, most showed no association with HF, with ~90% present in fewer than ~20% of iterations, indicating that this is a sparse prediction problem. Only proteins identified

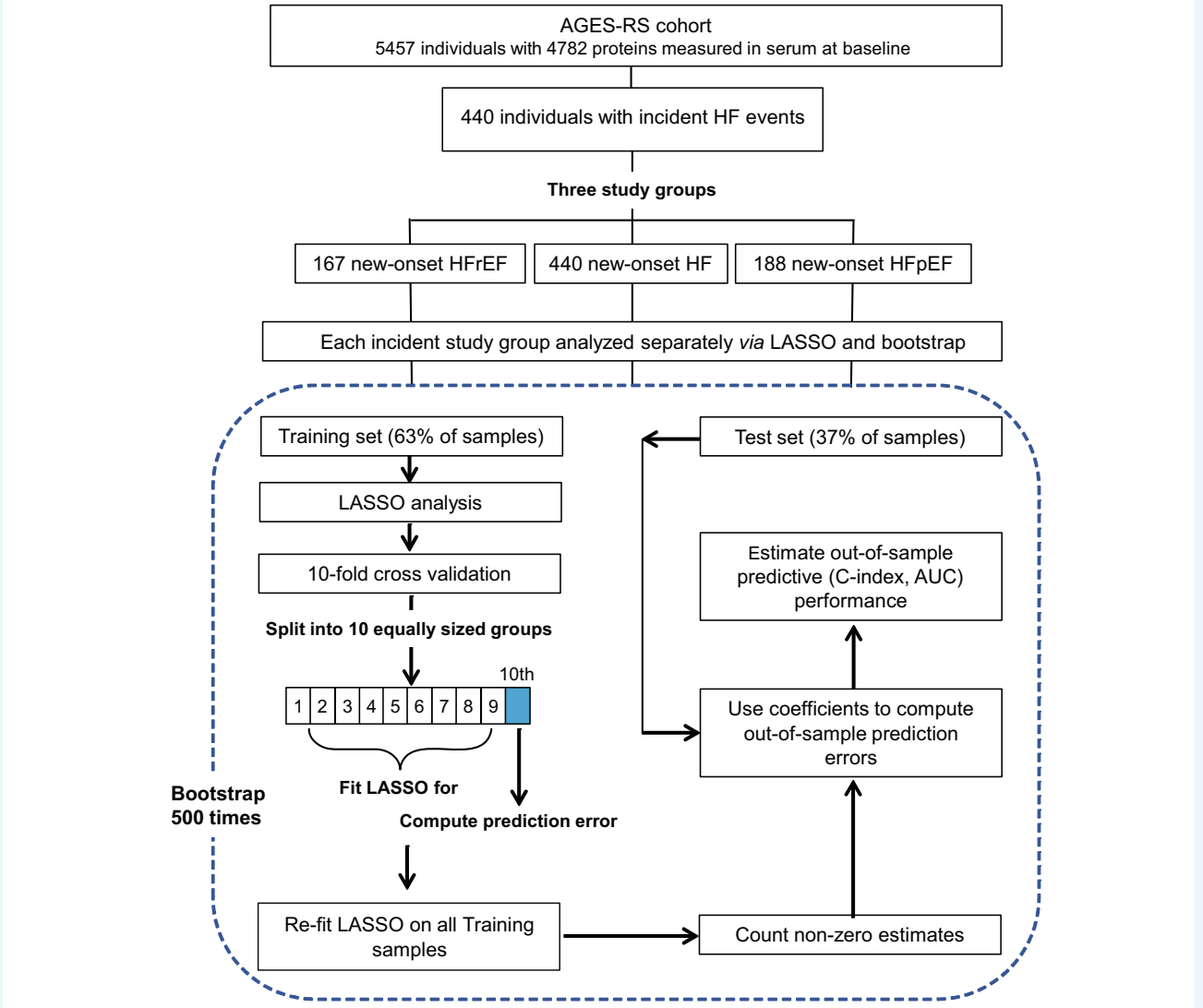


Figure 1 A flowchart describing the process of identifying protein predictors for future heart failure (HF) events in the Age, Gene/Environment Susceptibility Reykjavik Study (AGES-RS). The flowchart depicts the number of individuals in each HF group, as well as the approach and analysis that used least absolute shrinkage and selection operator (LASSO), non-parametric bootstrap, and internal cross-validation. For a more complete discussion, see *Methods*. The bootstrap validated concordance probability estimate (C-index) was based on out-of-sample predictions. All incident HF events, included all ejection fraction categories as well as those without echocardiographic data. AUC, area under the curve; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction.

in ≥80% of the bootstrap replications were evaluated further as potential predictors.

For incident HF, 10 proteins were selected, with NT-proBNP, troponin I3 cardiac type (TNNI3) and matrix metalloproteinase-12 (MMP12) found in over 99% of the iterations (online supplementary *Figure S1*). *Figure 2A* displays the concordance gain for individual proteins or all proteins combined as a weighted sum of measured coefficients (see *Methods*) for predicting incident HF events. Concordance gain is demonstrated using several models of adjustment incorporating various clinical variables and demographic information including the BMI-based Framingham risk score (FRS) components,[47] that is, age, sex, total cholesterol, HDL cholesterol, systolic blood pressure, smoking status and BMI, plus T2D as well as CAC, TAC and prior history of CHD. We used the FRS, as it is a well-established risk model for CHD and was originally developed for both CHD and HF,[48] and also because no single clinical risk model of HF has been fully validated and approved for use in the clinic.[49,50] Furthermore, adding a CAC score to the traditional FRS prediction model improved risk classification for future CHD events significantly.[51] The clinical characteristics that were most likely to predict all incident HF events using the LASSO model and 500 bootstrap iterations are shown in online supplementary *Figure S2*. It is evident that the protein predictors, either with or without NT-proBNP, improve prediction beyond the scope

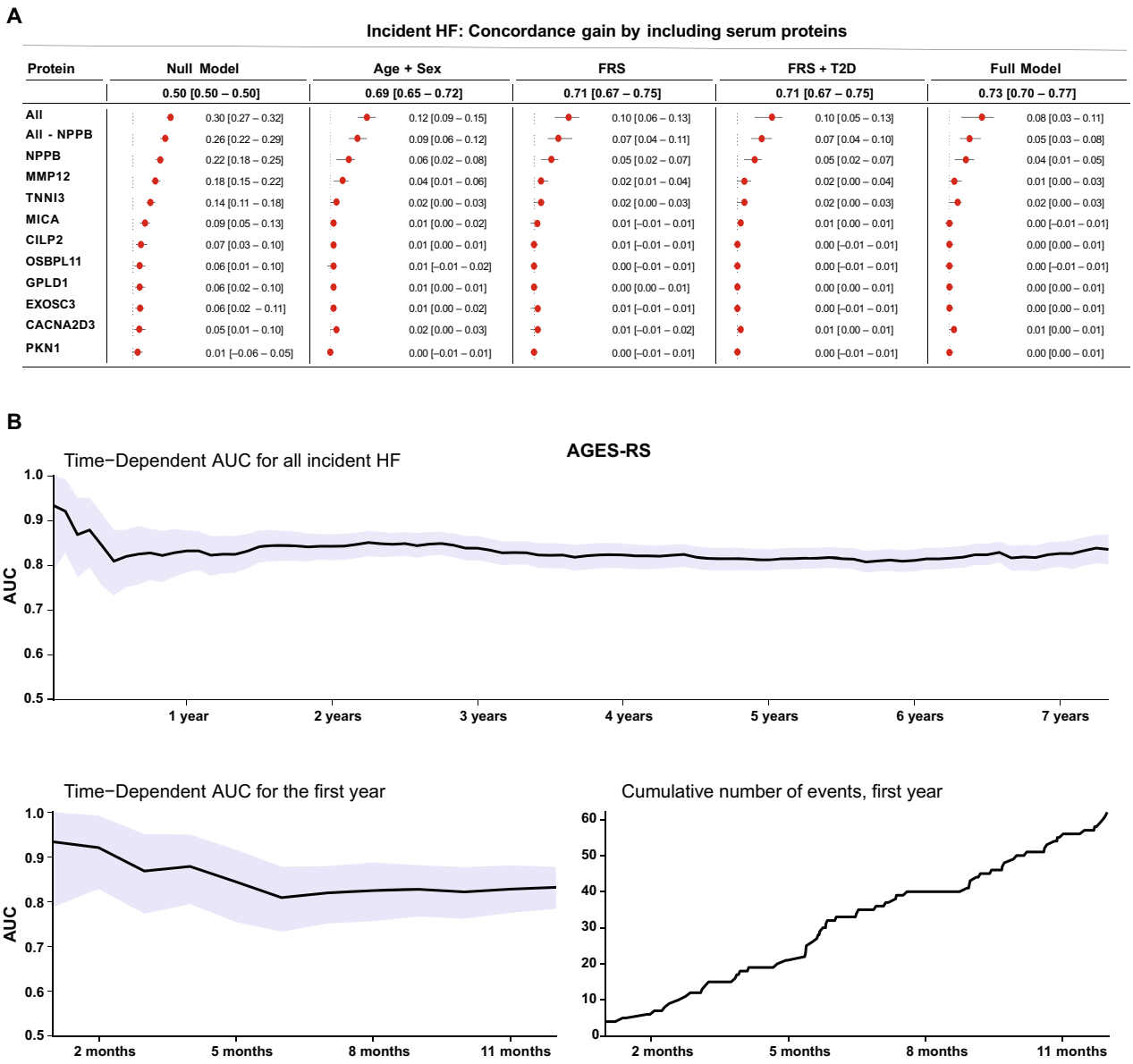


Figure 2 Serum protein predictors of all heart failure (HF) incidences in the Age, Gene/Environment Susceptibility Reykjavik Study (AGES-RS). (*A*) The numbers in each line represent the improvement in concordance (C-statistics) over chance in predicting incident HF, with 95% confidence intervals in brackets. There is no adjustment for any clinical variable in the null model. As we move to the right of the figure, we include age plus sex, then components of the body mass index-based Framingham risk score (FRS), FRS plus type 2 diabetes (T2D), and in the full model we adjusted for FRS, T2D, coronary heart disease, coronary artery calcium and thoracic aorta calcium. The protein predictors are highlighted individually and collectively in the leftmost column. The C-index for each model incorporating various clinical variables is presented in bold below each annotated model, along with 95% confidence intervals in brackets. In All (leftmost column), all 10 top proteins from the least absolute shrinkage and selection operator (LASSO) and bootstrap analysis are added to the model while in All-NT-proBNP, the NT-proBNP is removed. (*B*) An inverse probability of censoring weighting estimation of cumulative/dynamic time-dependent receiver operating characteristic curve (ROC) was used to compute the time-dependent area under the curve (AUC) at monthly intervals over a 8-year period (upper panel) in the AGES-RS, for prediction of all incident HF events. The prediction model did not include any demographic or clinical data. To estimate the uncertainty, we ran 500 bootstrap replications of the time-dependent ROC and calculated the mean, 2.5% quantile, and 97.5% quantile at each time point (blue shaded areas). The lower left panel displays the AUC versus HF event time for the first 12 months, while the lower right panel shows the cumulative number of HF events for the first 12 months.

of recognized clinical risk indicators (*Figure 2A*). All 10 proteins combined have a C-index of 0.80 in the null model, which includes no demographic or relevant clinical information (*Figure 2A*). This performance was superior to that of individual proteins, although MMP12 and NT-proBNP had high individual C-indices of 0.68 and 0.72, respectively (*Figure 2A*). *Figure 2A* also shows the C-index for each model with a broad range of demographic and clinical parameters but no proteins, demonstrating that protein predictor panels outperformed these models. The annual observed HF event rates and percentage survival for being diagnosed with HF during the follow-up period are displayed in online supplementary *Figure S3*.

Next, we calculated time-dependent AUC for the protein predictor panel using monthly intervals, over a nearly 8-year period, without including demographic or any clinical variables. To avoid chance observations, we estimated the uncertainty using 500 bootstrap iterations throughout the whole follow-up period, computing the mean, 2.5% quantile, and 97.5% quantile for each month (see *Methods*). Intriguingly, the protein panel performed best in the 5 months proximal to the event of HF with an AUC greater than 0.90 in the first 2 months and remaining at around 0.80 after 5 months and throughout the follow-up period (*Figure 2B*).

The performance of the protein predictors was evaluated over the first year of follow-up using alternative protein panel compositions (online supplementary *Figure S4*). Here, NT-proBNP predicts early HF events well (online supplementary *Figure S4A*), but the other nine proteins in the panel also predicted proximal disease well (online supplementary *Figure S4B*), with some additional improvements in performance, implying that these proteins may predict outcome independently of NT-proBNP. The protein predictors with the smallest individual estimates performed best near HF events, with overall lower aggregate estimates, as expected (online supplementary *Figure S4C*).

Serum proteins predict the incidence of the main subtypes of heart failure

We examined the ability of protein predictors and several clinical indicators to predict future HFrEF and HFpEF separately (online supplementary *Figure S5*). Online supplementary *Figure S5B* displays the serum proteins that have non-zero estimates in at least 80% of the bootstrap replications for incident HFrEF or HFpEF. *Figure 3A* shows the concordance gain for the protein predictors of HFrEF using several models of adjustment that incorporated various clinical variables of interest. Again, the protein panel has high prediction scores on its own and improves prediction beyond known clinical variables related to HF (*Figure 3A*). In the null model, that is no adjustment for clinical variables, all proteins combined have a C-index of 0.80 for HFrEF (*Figure 3A*), with individual protein predictors such as TNNI3, MMP12 and NT-proBNP predicting HFrEF with C-indices ranging from 0.67 to 0.74 (*Figure 3A*). *Figure 4A* depicts the improvement in concordance for the protein predictors of HFpEF for proteins alone and when various clinical variables are added to the prediction model. Here, the C-index of the combined protein panel predicting HFpEF was 0.78 (*Figure 4A*). Interestingly, NT-proBNP and MMP12 predicted HFpEF equally well, both with a C-index of 0.70 (*Figure 4A*), whereas tissue inhibitor of metalloproteinases 4 (TIMP4) had a C-index of 0.66. The protein TNNI3 did not appear in the HFpEF prediction panel. Also, it is worth mentioning that the variation of the individual protein predictor estimate sizes is greater for HFpEF than for HFrEF (*Figures 3A* and *4A*). The yearly observed event rates and percentage survival for being diagnosed with HFrEF or HFpEF during the follow-up period are shown in online supplementary *Figure S6*.

The protein predictor panel for HFrEF performed best proximal to the time of the event (*Figure 3B*). More specifically, the protein panel for HFrEF performed best in the 4 months preceding the HF event, with an AUC greater than 0.90 compared to 0.80 for the rest of the follow-up period (*Figure 3B*). Similar to HFrEF, the protein panel for incident HFpEF performed best close to event time, with AUCs exceeding 0.90 the first 2 months before an event (*Figure 4B*). The protein predictor panels for HFrEF and HFpEF outperform models that solely include traditional clinical and demographic factors, as shown in *Figures 3A* and *4A*.

Replication of the predictor time-dependent performance in the Cardiovascular Health Study cohort

For external validation, we used plasma proteome data and new-onset HF information from the prospective population-based CHS cohort.[42,44] The baseline characteristics of the CHS replication sample are provided in online supplementary *Table S1* and supplementary material. In order to focus solely on the protein predictors, the timeROC model, like in the AGES-RS analysis, did not include any information such as age and sex or known HF-associated clinical variables. The protein panel included all protein predictors identified in the AGES-RS for all incident HF ($n = 1198$) except for glycosylphosphatidylinositol-specific phospholipase (GPLD1), which was excluded during quality control in the CHS study (online supplementary material). We found that the AUC was greater than 0.90 in the first months and remained relatively high for the next 23 years with no signs of diminished effect (*Figure 5A*, online supplementary *Figure S7A*). Similarly, the protein predictor panels for incident HFrEF ($n = 316$) or HFpEF ($n = 360$) performed best near the time of the event and remained stable for up to 23 years of follow-up (*Figure 5B,C*, *Figure S7B,C*). The annual observed event rates and percentage survival for being diagnosed with all incident HF, HFrEF or HFpEF during the 23-year follow-up period are shown in online supplementary *Figure S8*.

Comparing the performance of the protein predictors to known heart failure risk factors

Table 2 compares the C-indices computed throughout the full follow-up period for various models incorporating demographic and clinical predictors (age, sex, NT-proBNP, FRS, etc.) alone or in combination with protein predictors for all incident HF. This comparison demonstrates that the newly found protein predictors significantly improve prediction of well-known HF risk variables,

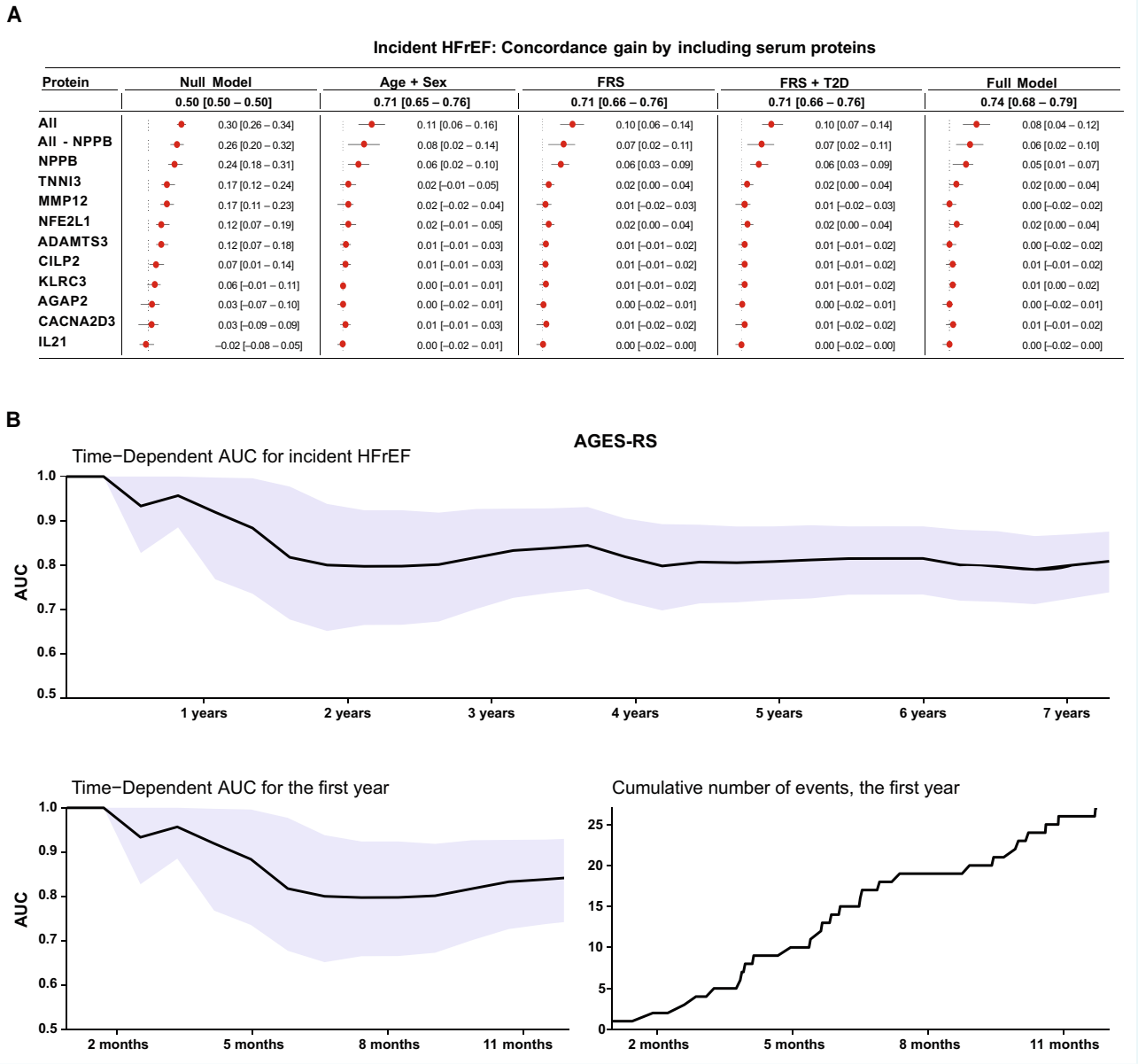


Figure 3 Serum protein predictors of incident heart failure with reduced ejection fraction (HFrEF) in the Age, Gene/Environment Susceptibility Reykjavik Study (AGES-RS). (*A*) The C-indices for the protein predictors of HFrEF, with a similar approach to that in *Figure* 2 (see description). The leftmost column displays individual protein predictors as well as all predictors combined. The full model adjusted for Framingham risk score (FRS), type 2 diabetes (T2D), coronary artery calcium plus history of coronary heart disease. In All (leftmost column), all 10 top proteins from the least absolute shrinkage and selection operator and bootstrap analysis are added to the model while in All-NT-proBNP, the NT-proBNP is removed. (*B*) Time-dependent receiver operating characteristic curve (ROC) for HFrEF in the AGES-RS based on the proteins alone from the corresponding prediction panel. The upper panels in each figure show time-dependent area under the curve (AUC) monthly over a 8-year period. To estimate the uncertainty, we ran 500 bootstrap replications of the time-dependent ROC and calculated the mean, 2.5% quantile, and 97.5% quantile at each time point (blue shaded areas). The upper left panel shows the AUC versus HF event time for the first 12 months, while the lower right panel shows the total number of HFrEF events for the first 12 months.

such as NT-proBNP, in the AGES-RS. Comparable to AGES-RS in terms of follow-up duration (8 years) and recognized risk variables, and focusing on all incident HF for statistical power, the inclusion of protein predictors resulted in higher C-indices in the CHS but was statistically significant only when compared to NT-proBNP and demographic factors (*Table* 2). It should be noted that one of the 10 protein predictors, GPLD1, was not included in the CHS cohort's protein prediction panel (online supplementary material). Further, the CHS has mixed ethnicity, which may have influenced the outcome of the replication effort in *Table* 2.

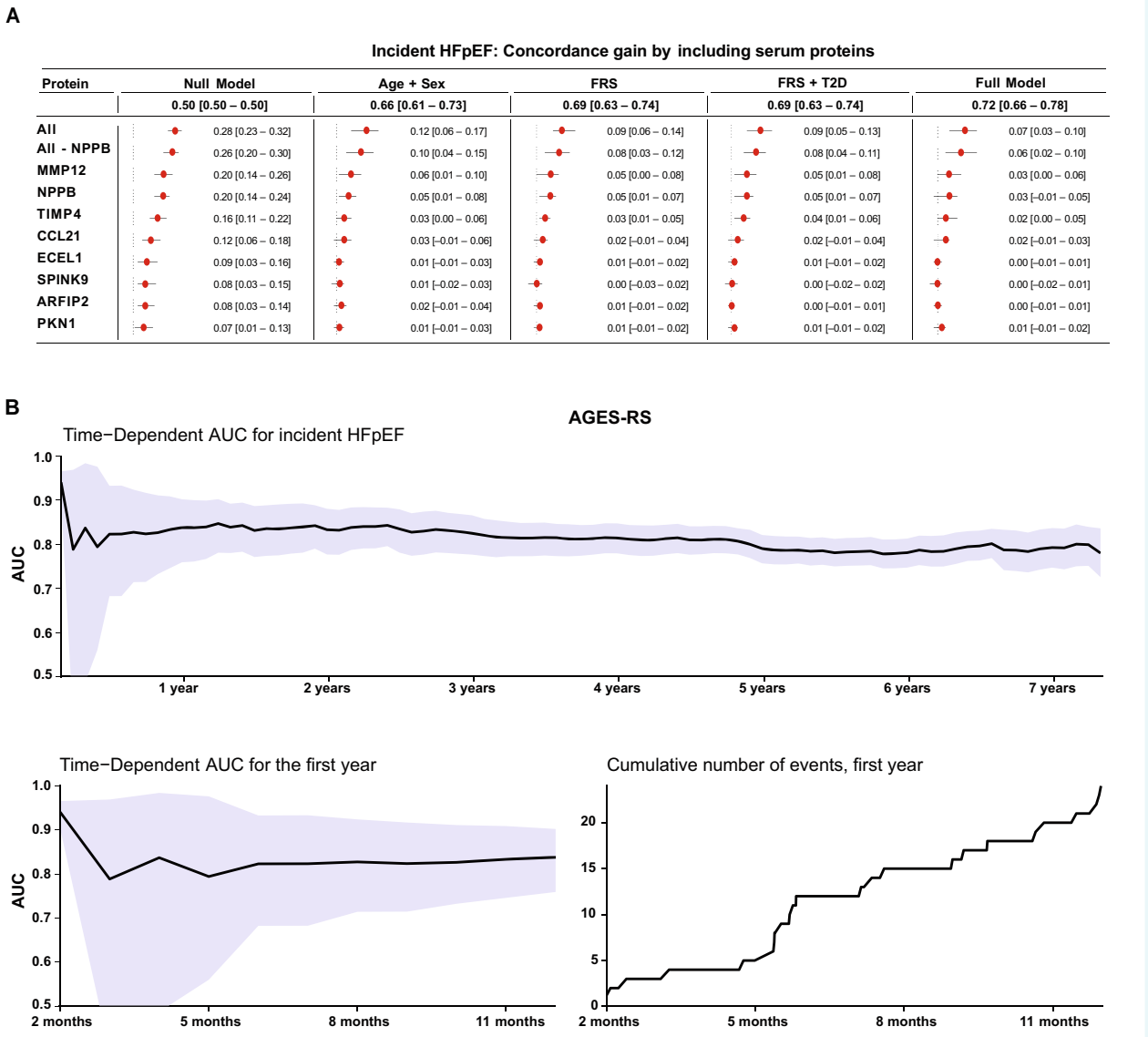


Figure 4 Serum protein predictors of incident heart failure with preserved ejection fraction (HFpEF) in the Age, Gene/Environment Susceptibility Reykjavik Study (AGES-RS). (*A*) The C-index for the protein predictors of incident HFpEF (see *Figure 2* for description). The leftmost column displays individual protein predictors as well as all predictors combined. In All (leftmost column), all eight top proteins from the least absolute shrinkage and selection operator and bootstrap analysis are added to the model while in All-NT-proBNP, the NT-proBNP is removed. In the full model we adjusted for Framingham risk score (FRS), type 2 diabetes (T2D) and thoracic aorta calcium. (*B*) AGES-RS time-dependent receiver operating characteristic curve (ROC) for HFpEF versus the corresponding protein predictor panel alone (no demographic or clinical information included). The upper panel shows time-dependent area under the curve (AUC) monthly over a 8-year period. To estimate the uncertainty, we ran 500 bootstrap replications of the time-dependent ROC and calculated the mean, 2.5% quantile, and 97.5% quantile at each time point (blue shaded areas). The lower left panel shows the AUC versus HF event time for the first 12 months, while the lower right panel shows the total number of HFpEF events for the first 12 months.

Characteristics of the proteins predicting future heart failure events

Figure 6 demonstrates the overlap in the number of shared or unique protein predictors for all incident HF, HFrEF, and HFpEF, totalling 20 distinct proteins. These proteins were significantly enriched for heart pathology-related processes, such as rheumatic heart disease, dilated cardiomyopathy, pericardial effusion, and heart atrial appendage (online supplementary material). Online supplementary material, *Appendix S1* and *Figures S9–S11* highlight protein predictors associated with incident HF and its subtypes using standard logistic regression analysis. NT-proBNP and MMP12 were selected by the LASSO model in all three incident HF analyses (*Figure 6*). Additionally, TINNI3 was included in the predictor

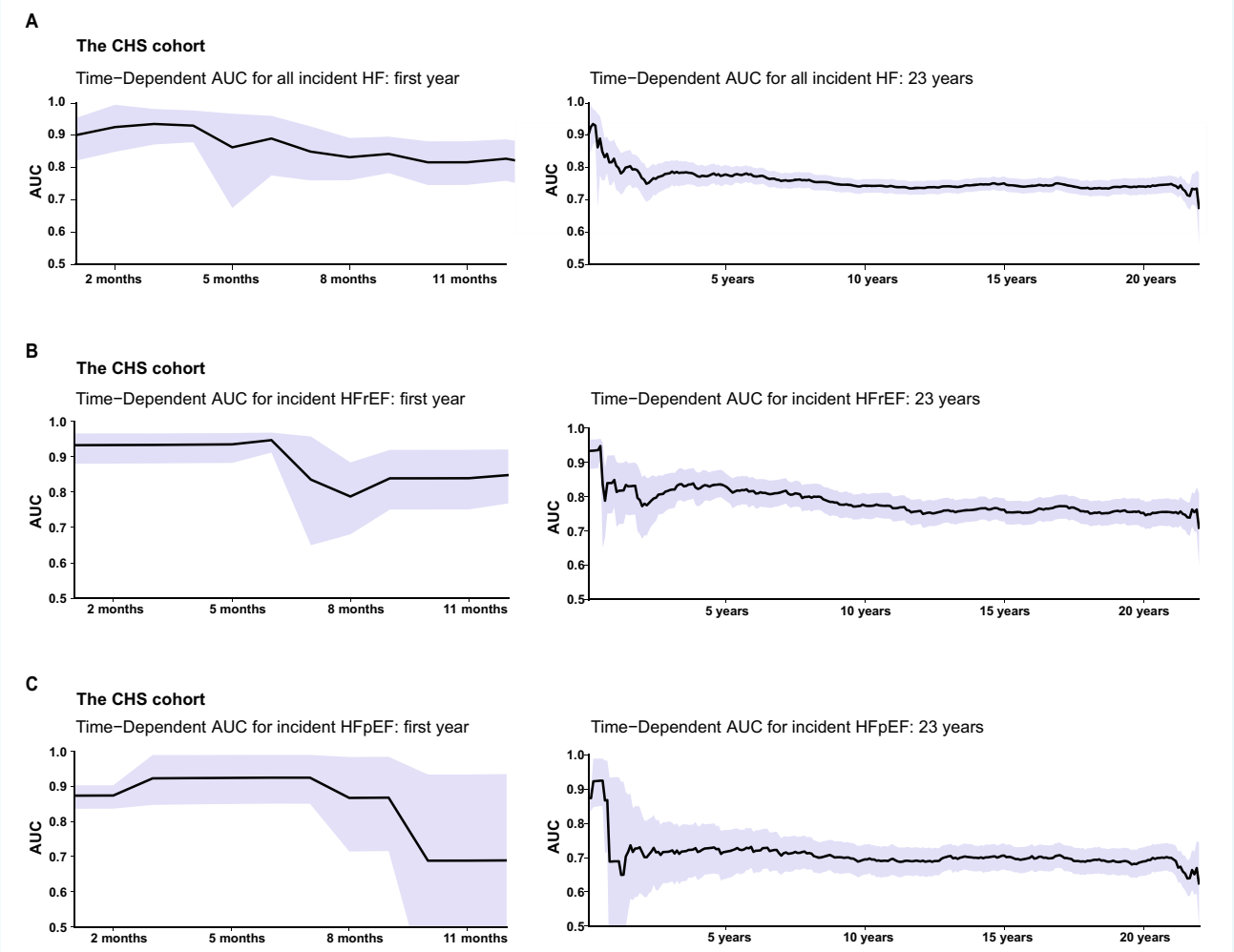


Figure 5 Replication of the prediction of the Age, Gene/Environment Susceptibility Reykjavik Study (AGES-RS) protein panels in the Cardiovascular Health Study (CHS) cohort. (*A*) Time-dependent receiver operating characteristic curve (ROC) for all incident heart failure (HF) ($n = 1198$), (*B*) incident heart failure with reduced ejection fraction (HFrEF) ($n = 316$) and (*C*) incident heart failure with preserved ejection fraction (HFpEF) ($n = 360$) in the prospective CHS (online supplementary material), showing the time-dependent area under the curve (AUC) versus event time for the first 12 months (left panels) and monthly over a 23-year period (right panels). To estimate the uncertainty, 500 bootstrap replications of the time-dependent ROC were carried out and the mean calculated with 2.5% and 97.5% quantiles at each time point (blue shaded areas). The prediction model did not include any demographic or clinical data. Online supplementary *Table S1* shows the total number of HF-related events in the CHS cohort throughout the first year.

panel for all incident HF and HFrEF but not HFpEF (*Figure 6*). In the AGES-RS, NT-proBNP, MMP12, and TNNI3 were directly associated with MI as well as CAC and TAC (online supplementary *Figure S12*, *Tables S3* and *S4*). NT-proBNP and MMP12 were also associated with carotid plaque (online supplementary *Figure S12*, *Tables S3* and *S4*), and unlike NT-proBNP and TNNI3, MMP12 was directly associated with T2D (online supplementary *Figure S12*, *Table S3*). Further, the proteins MHC class I polypeptide-related sequence A (MICA), C−C motif chemokine ligand 21 (CCL21), and killer cell lectin like receptor C3 (KLRC3) in the predictor panel of all incident HF or incident HFpEF were positively linked to CHD (online supplementary *Figure S12*, *Table S3*). CCL21 was also positively associated with T2D (online supplementary *Figure S12*).

In contrast, the cartilage intermediate layer protein 2 (CILP2) and calcium channel voltage-dependent, alpha 2/delta subunit 3 (CACNA2D3), in the prediction panels for all incident HF and HFrEF (*Figure 6*), protein kinase N1 (PKN1), ADP ribosylation factor interacting protein 1 (ARFIP), and serine peptidase inhibitor Kazal type 9 (SPINK9), all members of the HFpEF prediction panel (*Figure 6*), were found to be inversely related to T2D and various intermediate metabolic traits, but did not show links to MI or CHD (online supplementary *Figure S12*, *Tables S3* and *S4*). The extracellular matrix A disintegrin and metalloproteinase with thrombospondin type 1 motif 3 (ADAMTS3) of the prediction panel for HFrEF was inversely associated with both T2D and CHD (online supplementary *Figure S12*, *Tables S3* and *S4*). Finally,

Table 2 C-statistics for incident heart failure using several predictions with or without all protein predictors included

Model[a]	C-index for all incident HF events					
	AGES-RS cohort			CHS cohort		
	Mean	95% CI	*p*-value	Mean	95% CI	*p*-value
Age + sex	0.69	0.65–0.72		0.64	0.61–0.67	
Age + sex + NT-proBNP	0.74	0.71–0.78		0.71	0.69–0.76	
Age + sex + all proteins	0.80	0.77–0.83	<0.001	0.74	0.72–0.79	0.023
FRS	0.71	0.67–0.75		0.68	0.65–0.71	
FRS + NT-proBNP	0.76	0.73–0.80		0.74	0.72–0.79	
FRS + all proteins	0.80	0.77–0.83	0.001	0.76	0.74–0.81	0.058
Full model	0.73	0.70–0.77		0.71	0.69–0.76	
Full model + NT-proBNP	0.77	0.74–0.80		0.76	0.74–0.81	
Full model + all proteins	0.81	0.78–0.84	0.003	0.77	0.75–0.82	0.163

AGES-RS, Age, Gene/Environment Susceptibility Reykjavik Study; CHS, Cardiovascular Health Study; CI, confidence interval; FRS, Framingham risk score; HF, heart failure; NT-proBNP, N-terminal pro-B-type natriuretic peptide.

C-statistics are point estimates based on an 8-year follow-up period. The C-index was calculated in R using the concordance function from the survival package (function Surv). The non-parametric bootstrap was used to calculate the *p*-values empirically. In 1000 bootstrap iterations, the concordance values of models containing NT-proBNP versus all protein predictors were estimated, and the *p*-value calculated as the percent of iterations where the concordance for all protein predictors was greater than the concordance for NT-proBNP, i.e. a one-sided alternative to the null hypothesis of all protein predictors = NT-proBNP.

[a]FRS components; age, sex, total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, smoking status, and body mass index. The full model includes FRS + history of type 2 diabetes + history of coronary heart disease + coronary artery calcium + thoracic aorta calcium, and the term 'all proteins' is the same as shown in *Figure 2*.

the metalloproteinase inhibitor TIMP4, one of the leading predictors of HFpEF, was directly linked to plaque, CAC, and TAC (online supplementary *Figure S12*, *Tables S3* and *S4*). Many of these proteins were significantly associated with increased or reduced overall and/or post CHD survival (online supplementary *Table S3*), including for instance CILP2, SPINK9, TIMP4 and MMP12 (online supplementary *Figures S13* and *S14*). Other protein predictors were not associated with any or only a few disease-related traits (online supplementary material). In summary, these findings suggest a multifaceted basis of the protein predictor panels and HF outcomes.

Discussion

Circulating proteins reflect processes taking place in solid tissues, and many of them participate in cross-tissue regulatory loops, which could be a mechanism for system wide coordination in normal and disease settings including response to local (e.g. heart) pathological changes. Similarly, the onset of disease states in individual tissues is most likely the result of the interaction between local and global signals.[16,52] We examined the relationship of standard risk factors and the levels of thousands of serum proteins to future HF events in the AGES-RS cohort. This resulted in the identification of a small set of proteins that alone, or in combination with known risk factors, accurately predict incident HF events. Additionally, separate though somewhat overlapping sets of proteins could predict both incident HFrEF and HFpEF. Another notable finding is that the different protein predictor panels performed best for blood donors who were closest to the clinical diagnosis of HF, and remained relatively stable throughout the follow-up period. Importantly, the CHS, a population-based prospective cohort study of 1198 incident HF cases with a 23-year follow-up,[44,45] replicated this effect (*Graphical Abstract*).

The identified proteins appeared to capture the risk of new-onset HF linked with various clinical variables and risk not captured by those variables. All protein predictors improved AGES-RS prediction of HF significantly above NT-proBNP alone plus demographic and clinical variables (*Table 2*). When all protein predictors were compared to age, sex, and NT-proBNP alone, the C-statistics in CHS were significant (*Table 2*) but fell short when FRS and other clinical factors were included with NT-proBNP. We should point out that one of the proteins (GPLD1) was not included in the CHS panel (online supplementary material), and that the CHS cohort had mixed ethnicity, which could have influenced the replication outcome. More research will be needed to determine whether the existing multi-marker score performs better when restricted to populations of European ancestry only.

The current study demonstrates that both established protein biomarkers such as NT-proBNP and TNNI3 as well as additional new proteins contribute to the risk score in predicting future HF. For short-term HF prediction, these proteins may predominantly reflect preclinical HF (stage B) and incipient/mild clinical HF (stage C), whereas for long-term HF prediction, these proteins signal a prodromal stage encompassing stages A and B prior to clinically presenting HF. Identification of this high-risk group may allow for immediate evaluation and institution of lifestyle/pharmacologic interventions. Prevention trials like SOLVD,[53] STOP-HF,[54] and PONTIAC,[55] have shown improved patient outcomes when individuals with either asymptomatic LVEF,[53] or high plasma levels of BNP,[54] or NT-proBNP,[54] were identified before transitioning to symptomatic HF. Each of these prevention trials employed a therapeutic intervention that mostly consisted of renin–angiotensin

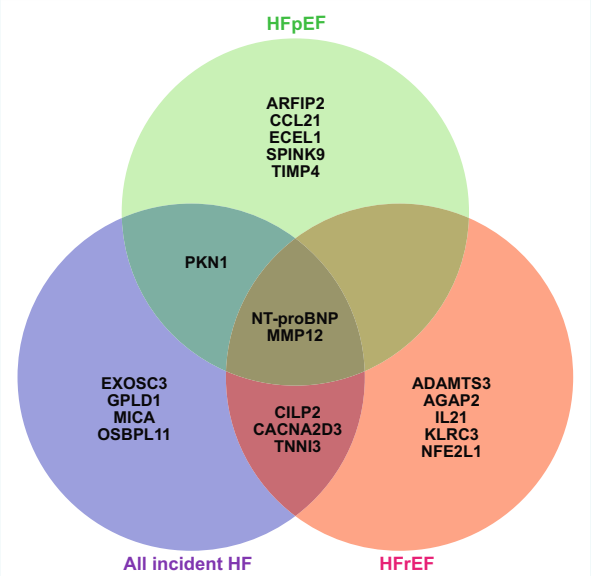


Figure 6 Venn diagram displaying the overlap between the various protein predictor panels. The various protein predictors that overlap or are unique for the different heart failure (HF)-related outcomes including all incident HF, incident HF with reduced ejection fraction (HFrEF), and/or HF with preserved ejection fraction (HFpEF), are listed in each relevant section of the diagram. The category of all event HF comprised all ejection fractions as well as participants lacking echocardiographic data.

system antagonists, and improved patient outcome was assessed via decreased hospitalization rate, lower rates of mortality, and a less severe left ventricular dysfunction. These trials show that early intervention can greatly lessen or delay the severity of HF-related consequences. The current study suggests that, in addition to NT-proBNP, other protein predictors identified for both short- and long-term prediction of future HF, including its major subtypes, may contribute to a more refined prediction and identification of high-risk individuals who may benefit from echocardiography and other testing for more accurate diagnosis and intensive intervention strategies. However, the benefit of employing multiple protein panels over NT-proBNP alone for identifying those at high risk for effective early intervention is beyond the scope of this study.

A total of 20 unique proteins, including NT-proBNP, were selected as predictors by the LASSO model for all incident HF, incident HFpEF, or incident HFrEF. NT-proBNP is currently the gold standard prognostic biomarker for HF,[1] so it is reassuring that it appears in all predictor panels of incident HF in our study. MMP12 appeared in all three incident HF analyses, while TNNI3 was one of the predictors for all incident HF and HFrEF events. MMP12 has previously been reported to predict incident HF events,[56] whereas TNNI3, a well-known plasma biomarker for acute MI,[57] has been associated with incident HF and cardiovascular-related mortality.[58] In the current study, all three proteins contributed strongly and independently to their respective prediction scores. TINNI3 did not appear in the prediction panel for incident HFpEF, but the metalloproteinase inhibitor TIMP4 did, with a similar estimate size to TINN3 for HFrEF. Interestingly, TIMP4 has been linked to heart tissue remodelling and HF in rodent models.[59] Proteins within the predictor panels with smaller estimates sizes than NT-proBNP, MMP12, TNNI3 and TIMP4, have been associated with HF-related outcomes that are directionally consistent with our findings including for instance CILP2 found with lower plasma levels in patients with diabetes and HFpEF,[60] PKN1 deficiency linked to systolic and diastolic dysfunction with preserved ejection fraction in a global ischaemia and reperfusion mouse model,[61] and CCL21 levels in plasma found to be directly related to survival in individuals with chronic HF.[62] Finally, NFE2L1, which is specifically expressed in cardiomyocytes, is activated in regenerating cardiomyocytes.[63] Many of the protein predictors represent well-known mechanisms driving HF, such as cardiac injury or remodeling (TNNI3, MMP12, NFE2L1, TIMP4), myocardial stretch (NT-proBNP), inflammatory processes (MICA, IL21, CCL21), extracellular matrix (CILP2, ADAMTS3), and unspecified cardiac dysfunction (PKN1). The roles of proteins like CACNA2D3, SPINK9, ARFIP2, GPLD1, ECEL1, EXOSC3, AGAP2, KLRC3 and OSBPL11 in HF-related pathology remain unclear. Finally, the wider range in protein predictor estimate sizes for HFpEF compared to HFrEF (*Figures 3A* and *4A*) may indicate that HFpEF is a more multifactorial syndrome. More research is needed before any conclusions can be drawn about the mechanistic relevance of the protein predictors to future risk of HF.

Risk prediction models are intended to identify people who are at high risk of developing a disease so that they can be targeted for additional testing and appropriate early intervention. The predictors themselves, on the other hand, do not necessarily imply a causal relationship with the outcome in the sense that modulating a predictor will affect the outcome.[64] Our findings lay the groundwork for identifying circulating protein and non-protein biomarkers that can predict future HF, including its main subtypes. As such they could be used in population surveillance to facilitate early identification of those at risk for HF and provide opportunities for monitoring interventions during the pre-clinical phase.

Limitations

This study has several limitations that must be acknowledged. The AGES-RS cohort is an older adult cohort with a multi-morbid profile that includes high prevalence of hypertension and CHD, and therefore at high risk of developing HF. This may limit the generalizability of prediction models to different populations. Furthermore, despite the fact that the LASSO model is designed to avoid overfitting, and that the discovery cohort used 500 repeats of 10-fold cross-validation and C-index based on out-of-sample predictions for internal validations, some of the estimates may be optimistic as they are obtained from the same sample. Although none have been fully validated, numerous risk prediction models for HF have been established,[65] which used risk factors not included in the current study, such as urinary albumin/creatinine ratio, heart rate and atrial fibrillation, to name a few. The presented findings were limited to serum proteins and may not fully capture HF-related pathobiology in solid tissue such as the heart. Moreover, the study does not examine the entire serum proteome, which is still being identified.

Some of the serum protein predictors for HF in this study are characterized as intracellular proteins, and the significance of their presence in serum, remains to be determined. Finally, as all AGES-RS and majority (~80%) of CHS participants are of European ancestry, the results' transferability and generalizability needs to be tested across all ethnicities.

Conclusions

We examined 4782 circulating proteins in serum as well as multiple clinical variables measured in the deeply annotated AGES-RS population-based cohort for prediction of incident HF. A small subset of serum proteins emerged as independent predictors of incident HF, including new-onset HFpEF and HFrEF, complementing previously approved clinical biomarkers like NT-proBNP and the FRS components. Intriguingly, the protein predictors performed particularly well near the event time, a finding that was replicated in the CHS cohort, a population-based prospective study of older adults, implying opportunities for early diagnosis and immediate intervention. This study offers a unique opportunity for further validation of the protein predictor panel presented, panel expansion with new proteins measured, leading to the development of an accurate and robust prediction panel that, in one platform, in conjunction with currently approved diagnostic tools, could be used by clinical practitioners for early diagnosis and prediction of new-onset HFpEF and HFrEF. The single platform nature of the measures may allow for more extensive at-risk population surveillance by physicians, as well as the possibility of early imaging and intervention in advance of clinical onset of HF.

Supplementary Information

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

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