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Multi-Proteomic approach to predict specific cardiovascular events in patients with diabetes and myocardial infarction: findings from the EXAMINE trial

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Abstract

Background: Patients with diabetes who had a recent myocardial infarction (MI) are at high-risk of cardiovascular events. Therefore, risk assessment is important for treatment and shared-decisions. We used data from EXAMINE trial to investigate whether a multi-proteomic approach would provide specific proteomic signatures and also improve the prognostic capacity for determining the risk of cardiovascular death, MI, stroke, heart failure [HF], all-cause death, and combinations of these outcomes.

Methods: 93 circulating proteins (92 from the Olink® CVDII plus troponin) were assessed in 5,131 patients. Cox, competing-risks, and reclassification measures were applied.

Results: The clinical model showed good discrimination and calibration for all outcomes. On top of a well calibrated clinical model that included age, sex, smoking, diabetes duration, history of MI (prior to the index MI of inclusion), history of HF hospitalization, history of stroke, atrial fibrillation, hypertension, systolic blood pressure, statin therapy, estimated glomerular filtration rate, and study treatment (alogliptin or placebo), troponin and BNP added prognostic information to the composite of cardiovascular death, MI, or stroke (Δ C-index +5%) and cardiovascular death alone (Δ C-index +7%). Troponin, BNP, and TRAILR2 added prognostic information on all-cause death and the composite of cardiovascular death or HF-hospitalization. HF-hospitalization alone was improved by adding BNP and Gal-9. For MI, troponin, FGF23, and AMBP added prognostic value, whereas for stroke only troponin added prognostic value (multi-proteomics improved C-index >3% [p<0.001] for all the studied outcomes). The addition of the final biomarker selection to the clinical model improved event reclassification (cNRI from +23% to +64%). Specifically, the addition of the biomarkers allowed a better classification of patients at low risk (as having “true” low risk) and patients and high risk (as having “true” high risk). These results were consistent for all the studied outcomes with even more marked differences in the fatal events.

Conclusions: The addition of multi-proteomic biomarkers to a clinical model in this population with diabetes and a recent MI, allowed a better risk prediction and event reclassification, potentially helping for better risk assessment and targeted treatment decisions.

Key-words: proteomics; diabetes; cardiovascular events.

Background

The prevalence of type 2 diabetes is increasing worldwide; currently over 500 million adult people may have diabetes and this number will likely increase in the next decades¹. Per year over 5 million people die due to complications of diabetes¹. Beyond the fatal complications, diabetes is an important risk factor for the occurrence of non-fatal major adverse cardiovascular events (MACE), such as myocardial infarction (MI), stroke, and heart failure (HF)². The occurrence of these events increases the risk of subsequent death and impairs quality of life, frequently accompanied by significant disability.

In patients with diabetes, intensive intervention with drug combinations and behavioural changes reduced cardiovascular complications and death³. Strategies for improving event prediction may be useful to implement preventive measures tailored to patients at higher risk for specific events; to attain this purpose one requires a better classification of risk. For example, knowing which patients are more likely to develop events such as HF, MI, or stroke may help in implementing strategies such as a more strict blood-pressure control, use of sodium-glucose cotransporter-2 inhibitors^{4,6}, aggressive statin therapy and/or PCSK9 inhibitors^{7,8}, and/or non-VKA anticoagulants^{9,10}.

The addition of biomarkers (e.g., natriuretic peptides and troponin) to patients' clinical features has proven to add relevant prognostic information in many populations, including in patients with diabetes and coronary artery disease¹¹⁻¹⁴. However, the application of a large proteomics multimarker panels (including BNP and troponin) to study the occurrence of specific cardiovascular events in patients with type 2 diabetes and a recent MI is yet to be performed. Such approach may add relevant (clinical and research) information, about the prognosis and mechanisms associated with specific cardiovascular events.

The Examination of Cardiovascular Outcomes with Alogliptin versus Standard of Care (EXAMINE) trial sought to determine whether alogliptin was noninferior to placebo with respect to major cardiovascular events in patients with type 2 diabetes with recent acute coronary syndrome^{15,16}.

We used the EXAMINE trial data to investigate whether a multi-proteomic approach comprising 93 circulating proteins would: 1) present different proteomic signatures for each

cardiovascular outcome, and 2) if these proteins could improve the discrimination of the outcomes (cardiovascular death, MI, stroke, HF) and death on top of a well calibrated risk model.

Methods

Study design

Details of the EXAMINE study design were previously published^{15, 17}. A total of 5380 patients were randomized to either alogliptin or placebo. The median age was 61 years, 68% were male, the mean body mass index was 29 Kg/m², and the median estimated glomerular filtration rate was 71 ml/min/1.73m². In the overall population, alogliptin significantly reduced glycated hemoglobin without increasing the rates of major adverse cardiovascular events.

For the present analysis 5131 patients (95% of the total population) who had biomarker measurements were included.

The steering committee, consisting of academic members and three nonvoting representatives of the sponsor (Takeda Development Center Americas), designed and oversaw the conduct of the trial. An independent data and safety monitoring committee monitored the trial and had access to the unblinded data. The appropriate national and institutional regulatory authorities and ethics committees approved the study design, and all participants provided written informed consent.

Data and Resource Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Study patients

Patients were eligible for enrolment if they had received a diagnosis of type 2 diabetes mellitus, were receiving antidiabetic therapy (other than a DPP-4 inhibitor or GLP-1 analogue), and had had an acute coronary syndrome within 15 to 90 days before randomization. Further criteria for the diagnosis of type 2 diabetes included a glycated hemoglobin level of 6.5 to 11.0% at screening, or if the antidiabetic regimen included insulin, a glycated hemoglobin level of 7.0 to 11.0%. Acute coronary syndromes included acute myocardial infarction and unstable angina requiring hospitalization. Major exclusion criteria were a diagnosis of type 1 diabetes, unstable cardiac disorders (e.g., New York

Heart Association class IV heart failure, refractory angina, uncontrolled arrhythmias, critical valvular heart disease, or severe uncontrolled hypertension), and dialysis within 14 days before screening.

Institutional Review Board approval was obtained and all patients provided informed consent to participate in the trial. The EXAMINE trial is registered in ClinicalTrials.gov with the number NCT00968708.

Study treatment

Patients were randomly assigned to receive alogliptin or placebo, administered in a double-blind fashion, in addition to standard-of-care treatment for type 2 diabetes mellitus. Throughout the study, patients were required to receive standard-of-care treatment for type 2 diabetes and cardiovascular risk factors according to regional guidelines.

End points

The primary endpoint was a composite of death from cardiovascular (CV) causes, nonfatal MI, or nonfatal stroke. For the present analysis we also studied HF endpoints, including a composite of CV death or HF hospitalization, and each individual component of the primary outcome, as well as all-cause mortality (ACM). The endpoint of hospital admission for heart failure (HF) was defined as an inpatient admission or an emergency department visit of more than 12h with clinical manifestations of HF. An independent central adjudication committee adjudicated all suspected primary endpoint events and other cardiovascular endpoints, as well as all deaths. The median follow-up time was 1.6 (1.0-2.2) years.

Clinical and biomarker measurements

Medical history and a physical examination were all recorded at baseline.

A large protein biomarker panel with 92 biomarkers from a wide range of pathophysiological domains was measured (Olink® CVDII panel) at baseline (randomisation). We selected this panel because it contains known human cardiovascular and inflammatory markers as well as some exploratory human proteins which may have potential as new markers of cardiovascular disease. These proteins are involved in multiple disease processes such as immune/inflammatory response, angiogenesis, coagulation and cell adhesion, for example. An overview of biomarkers, their full names, and Uniprot ID are presented in the *supplemental table 1*. In short, the biomarkers were

measured using a high-throughput technique using the Olink Proseek® Multiplex CVD II 96x96 kit, which measures 92 manually-selected cardiovascular-related proteins simultaneously in 1 µl plasma samples. The kit uses a proximity extension assay (PEA) technology, where 92 oligonucleotide-labeled antibody probe pairs are allowed to bind to their respective target present in the sample. PEA is a homogeneous assay that uses pairs of antibodies equipped with DNA reporter molecules. When binding to their correct targets, they give rise to new DNA amplicons each ID-barcoding their respective antigens. The amplicons are subsequently quantified using a Fluidigm BioMark™ HD real-time PCR platform. The platform provides Log₂ normalized protein expression (NPX) data wherein a high protein value corresponds to a high protein concentration, but not an absolute quantification. For further details please see the *supplemental Olink® addenda*. In addition to the above referenced Olink® biomarkers, high-sensitivity troponin I (hsTnI; troponin) was measured on the ARCHITECT i2000SR analyser (Abbott Laboratories) for a total of 93 circulating biomarkers.

Statistical considerations

The selection of the variables to include in the clinical model was based on their clinical relevance and previous publications from the EXAMINE dataset¹⁸⁻²⁰, and included age, sex, smoking, diabetes duration, previous MI, previous HF hospitalization, previous stroke, atrial fibrillation (AFib), hypertension, systolic blood pressure, statin therapy, estimated glomerular filtration rate (eGFR based on the CKD-EPI formula²¹), and study treatment (alogliptin or placebo) allocation. These variables were used for adjustment in all the models (i.e. “forced” in the models) and the strength of their association with the study primary outcome is depicted in the **Table 2**, where the variables are ordered by their z-score (from higher to lower) after fitting a Cox model. The clinical model was well calibrated (goodness of fit test p-value >0.10 for all the studied outcomes; *supplemental figure 1*) and showed moderate to good discrimination for all the outcomes (c-index ranging from 0.66 to 0.79 as described in the legend of the **Table 2**). On top of the clinical model we assessed the prognostic association of each individual biomarker correcting for false discoveries with a “false discovery rate” (FDR) of 0.01 as described by Benjamini and Hochberg²². Only the biomarkers that passed this 1% threshold (plus clinical covariate adjustment) were taken forward to the next steps. These results are fully shown in the *supplemental tables 3 to 9*. Taking the FDR1% biomarker selection, we then

performed a multivariable stepwise forward selection model with a p-value set at 0.05 for the biomarkers to stay in the model (with the clinical variables “forced” in the model). These results are shown in the **Table 3**, where the biomarkers are ordered by decreasing value of their z-score. These selected biomarkers were poor to moderately correlated (Spearman Rho ≤ 0.5 , with exception of TRAILR2 and AMBP with a Spearman Rho =0.6. *Supplemental table 10*). To further select which biomarkers added prognostic information on top of the clinical model, we assessed the Harrel’s C-statistics (“c-index”) and the Bayesian Information Criterion (BIC) for each biomarker in a sequential fashion starting with a clinical model alone and adding each biomarker by descending order of its prognostic strength (z-score) i.e. clinical model + biomarker 1, then clinical model + biomarker 1 + biomarker 2, etc. The C-statistic gives the probability that a randomly selected patient who experienced an event had a higher risk score than a patient who had not experienced the event²³. When fitting a model, it is possible to increase its likelihood by adding parameters (e.g. biomarkers), but doing so may result in overfitting; the BIC attempts to resolve this problem by introducing a penalty term for the number of parameters in the model, where the model with the lowest BIC is considered the best²⁴. We then plotted the C-index and the BIC for each outcome by sequentially adding each biomarker and stopping when the C-index no longer improved and the BIC was at a minimum i.e. when the addition of more biomarker did not improve the c-index nor the BIC at a p-value threshold of 0.05. These results were consistent for all the outcomes and are presented in the **Figure 1**, *supplemental figure 2*, and **Table 3** (blue cells). We then assessed the Net Reclassification Improvement (NRI) added by this “final” biomarker selection biomarkers on top of the clinical model by performing a 10-fold cross-validation with 1000x bootstrap NRI statistics. The NRI attempts to quantify how well the new model (e.g. clinical+biomarker) correctly reclassifies patients compared with the old model (e.g. clinical only). The NRI is composed of two components: 1) events and 2) no-events. Patients with (and without) events who were correctly reclassified by the new model are assigned a +1, and those who were incorrectly classified are assigned a -1. The sum of these two values is the NRI²⁵. These results are presented in the **Table 4** (a concrete example of NRI interpretation is given in the legend of the Table 3). We then built a risk model derived from the linear predictor of each model (clinical or clinical plus biomarker) for each outcome, where each dependent

variable is multiplied by its β coefficient and then divided in quintiles with similar number of patients. Events and event rates per 100 patient-years were assessed by each quintile of risk based on the clinical risk model and the clinical plus biomarker model, independently. These results are presented in the **Table 5** and plotted as a continuous function in the **Figure 2**. The patients' characteristics by quintiles of risk are presented in the *supplemental table 11*. Survival analysis were performed using Cox (for the primary outcome, CV death or HF hospitalisation, CV death and all-cause death) and competing-risk models (for HF hospitalizations, MI, and stroke) as described by Fine and Gray²⁶, using death as competing risk. We have also performed the analysis of the top biomarkers stratified by sex to assess whether the prognostic values of these markers could vary by sex. For internal validation purposes we have also performed Least Absolute Shrinkage and Selection Operator (LASSO) with 10-fold cross validation model using 70% of the sample as training and 30% as test for the study primary outcome. All statistical analyses were performed with STATA (version 15).

Results

Patient characteristics

A total of 5131 patients were included in this study. The mean age was 61 ± 10 years, 68% were male, the mean eGFR was 71 ± 21 ml/min/1.73m², the mean systolic blood pressure was 129 ± 17 mmHg, and 30% were treated with insulin.

The study primary outcome occurred in 590 (11.5%) of the patients, the composite of CV death or HF hospitalization in 377 (7.3%), all-cause death in 302 (5.9%), CV death in 226 (4.4%), non-fatal MI in 345 (6.7%), non-fatal stroke in 60 (1.2%), and HF hospitalization in 185 (3.6%).

Supplemental table 2.

Patients who had an event were older, had longer diabetes duration, hypertension, prior MI, HF, stroke, peripheral artery disease, and atrial fibrillation more often, lower eGFR, and higher total and LDL cholesterol. **Table 1.**

Clinical model

The clinical model presented in the **Table 2** was well calibrated (*supplemental figure 1*) and showed good discrimination for most of the studied outcomes: c-index =0.68 for the primary outcome of CV

death, MI, or stroke; c-index =0.75 for CV death; c-index =0.74 for all-cause death; c-index =0.76 for CV death or HFH; c-index =0.79 for HFH; c-index =0.66 for MI; and c-index =0.70 for stroke.

Biomarker selection

The biomarker associations with the study outcomes is shown in the *supplemental tables 3 to 9*. Of these, troponin, BNP (brain natriuretic peptide), TRAILR2 (trail receptor 2 or death receptor 5), VEGFD (vascular endothelial growth factor D), FGF23 (fibroblast growth factor 23), and MMP7 (matrix metalloproteinase 7) were independently associated with the primary outcome after the stepwise selection; with troponin and BNP adding relevant prognostic information to the multivariable model (Δ C-index +5%, $p<0.001$). For CV death the biomarkers with independent prognostic association were, BNP, troponin, TRAILR2, CEACAM8 (carcinoembryonic antigen related cell adhesion molecule 8) and VEGFD (vascular endothelial growth factor D); with BNP and troponin adding prognostic value (Δ C-index +7%, $p<0.001$). For all-cause death the biomarkers with independent prognostic association were, BNP, troponin, TRAILR2, CEACAM8, ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) and TF (transferrin); with BNP, troponin and TRAILR2 adding prognostic value (Δ C-index +6%, $p<0.001$). For the composite of CV death or HF hospitalization the biomarkers with independent prognostic association were, BNP, troponin, TRAILR2, VEGFD, and Gal-9 (galectin-9); with BNP, troponin and TRAILR2 adding prognostic value (Δ C-index +4%, $p<0.001$). For the outcome of HF hospitalization alone the biomarkers with independent prognostic association were, BNP, Gal-9, VEGFD, FGF23, troponin, SCF (stem cell factor), GIF (gastric intrinsic factor); with BNP and Gal-9 adding prognostic value (Δ C-index +17%, $p<0.001$). For the outcome of MI, the biomarkers with independent prognostic association were, troponin, FGF23, AMBP, and MMP7; with troponin, FGF23 and AMBP adding prognostic value (Δ C-index +3%, $p<0.001$). For the outcome of stroke, the biomarkers with independent prognostic association were, troponin and ADAMTS13; with troponin adding prognostic value (Δ C-index +3%, $p<0.001$). **Table 3, Figure 1, and supplemental figure 2.** No biomarker-by-sex interaction was present (p for interaction >0.1 for all the studied outcomes).

Adding LDL cholesterol and c-reactive protein to the adjustment variables provided similar results, as did a 10-fold cross-validation LASSO for the primary outcome with 70% of the population as training and 30% test sets (*supplemental appendix*).

Event reclassification

The addition of the final biomarker selection (biomarkers marked “blue” in the **Table 3**) to the clinical model, held significant event reclassification, where both events and non-events were better classified by adding the selected biomarkers, with continuous NRI global improvement ranging from 23% to 64%. **Table 4**.

Events and clinical utility

Aligned with the above, the addition of the biomarkers allowed a better classification of patients at low risk (as having “true” low risk) and patients and high risk (as having “true” high risk). **Table 5**. For example, by dividing the patients` risk in quintiles of risk the primary outcome event rate was of 2.6 and 4.4 events per 100py for the lowest 2 quintiles of risk (i.e. “low” risk) in the clinical model, compared with 2.0 and 3.5 events per 100py by adding the selected biomarkers. On the other hand, patients in the highest risk quintile had 18.6 events per 100py using the clinical model vs. 19.9 events per 100py by adding the selected biomarkers. These results were consistent for all the studied outcomes with even more marked differences in the fatal events. This prognostic improvement can be visualized in the **Figure 2**.

By using the biomarker models (as compared with the clinical models alone), younger patients and with less co-morbid conditions could be classified as “high risk”. *Supplemental table 11*.

The summary of these findings is depicted in the **Graphical Abstract**.

Discussion

Our study shows that different CV outcomes may have different proteomic signatures and that BNP and troponin (the most widely used and validated biomarkers) were not retained in all the studied outcomes. Moreover, the addition of biomarkers to the clinical model in a population with diabetes and a recent MI, allows a better risk prediction and event reclassification, potentially identifying younger patients and with less comorbidities that are at higher risk than what would be apparent if one

would only use the clinical variables. These findings may have clinical implications by identifying patients with a greater margin for aggressive treatment with potential life-saving implications. To the best of our knowledge this is also the first large outcome study in among post-MI patients with diabetes using a large panel of biomarkers potentially related to cardiovascular disease.

A previous analysis of the EXAMINE trial demonstrated that troponin (baseline and serial) has independent prognostic value in this population²⁰. Troponin had a linear relationship with the studied cardiovascular outcomes, whereby even small elevations in this biomarker should raise alert. The strong and independent prognostic value of troponin in patients with and without diabetes and/or coronary artery disease has been extensively replicated and supports both the internal and external robustness of our findings^{14,27}. Troponin added prognostic value to all the studied outcomes, except HF hospitalization. Along with troponin, BNP was the other biomarker that showed a linear, strong and independent association with all the studied outcomes, except for MI and stroke where troponin was clearly more relevant; on the other hand, BNP was strongly associated with the occurrence of HF hospitalizations. In a previous report from the EXAMINE data, NT-pro BNP was the biomarker with the strongest association with HF hospitalization²⁸. One may then suggest that these two biomarkers (BNP/NT-pro BNP and troponin) should be measured in all patients with diabetes with a MI. These two markers refined the prognosis in all the studied cardiovascular outcomes and also for all-cause death. Moreover, along with troponin, the strong and independent associations of natriuretic peptides with outcomes, have been replicated in patients with and without diabetes and/or coronary artery disease, adding important consistency to our findings^{13,16,29}. On top of troponin and BNP, TRAILR2 came out as a strong and independent prognosticator for all-cause death and the composite of HF hospitalization or CV death. The TRAILR2 protein (otherwise known as death receptor 5) is a member of the TNF superfamily encoded by the *TNFSF10* gene and is a receptor that preferentially induces apoptosis after binding of its ligand TRAIL^{30,31}. Increased levels of TRAILR2 have been associated with fatal events in patients with MI, possibly due to intensified apoptotic activity driven by TRAIL/TRAILR2^{32,33}. It was recently found that TRAILR2 was also strongly associated with new-onset HF via mechanisms linked to inflammation and apoptosis³⁴, once again reinforcing the robustness of these findings. Additionally, we also found that Gal-9 was strongly and independently

associated with HF hospitalizations, and FGF23 plus AMBP with MI, improving the prognostic models of the respective outcomes. Gal-9 is produced by the extracellular matrix and may be increased in patients with ischemic cardiovascular disease³⁵. Gal-9 has also been previously identified as strongly associated with new-onset HF³⁴, via mechanisms linked to fibrosis and extra-cellular matrix remodelling. FGF23 is released by the osteocytes and is essential for the regulation of the metabolism of phosphate, calcium and vitamin D. Importantly, FGF23 promotes myocardial fibrosis and has been associated with coronary heart disease and HF^{34, 36, 37}. Increased expression of AMBP has been associated with atherosclerotic cardiovascular disease and increased levels of c-reactive protein in patients with diabetes^{38, 39}.

Troponin and/or BNP consistently added prognostic value across all the studied outcomes (BNP and troponin for the primary outcome, CV death and all-cause death; BNP alone for HF hospitalization; troponin alone for non-fatal MI and stroke), meaning that ischemia and myocardial stretch/heart failure are major drivers of cardiovascular outcomes in post-MI patients with type 2 diabetes. Therefore, strategies to reduce the burden of ischemia and myocardial stretch may improve outcomes in this population. These findings are supported by recent trials in patients with diabetes, where SGLT2-inhibitors and GLP-1 agonists reduced cardiovascular events possibly mediated by volume depletion and by myocardial ischemia reduction, respectively^{40, 41}.

Beyond the prognostic and mechanistic implications of our findings, the use of biomarkers allowed a better reclassification of the outcomes. These findings are of particular relevance, because the use of biomarkers (on top of the clinical variables) allowed the classification of younger patients and with less comorbidities as “high-risk”; these younger patients may thrive more long-term benefit from aggressive treatment of diabetes and other cardiovascular conditions (e.g. hypertension, dyslipidaemia). Current risk stratification strategies and guidelines do not routinely incorporate biomarkers, such as troponin and NT-pro BNP, for improving the prediction of risk in patients with diabetes^{42, 43}. Our findings support a more widespread use of biomarkers for allowing a better risk prediction and event reclassification.

Limitations

Several limitations should be acknowledged in this analysis. First, this is a post-hoc analysis of a prospective randomized trial, therefore all limitations inherent to such analysis are applied herein, including the inability to infer causality. Second, with exception of troponin, these biomarkers are expressed in arbitrary Log2 normalized NPX units (as detailed in the methods section and supplemental material), therefore the inference of a ready-to-use clinical score is not possible at this stage, and the hazard ratios for each protein estimate the increase in the hazards of outcome associated with a doubling in the protein concentration. Third, we measured only one panel (CVDII) of 92 circulating proteins plus troponin; hence, we cannot ascertain whether a wider set of circulating proteins would provide additional information. Fourth, we did not perform external validation of our findings because we did not have access to a database/population with similar characteristics and with the same biomarkers. Despite not having such a replication cohort, we did random sampling and bootstrap analysis with internally consistent results. More importantly, all the biomarkers we found have been replicated in other studies, including cohorts with patients with and without diabetes. Notwithstanding, the absence of formal external replication is a limitation in our study⁴⁴. Fifth, comparing our findings with a cohort of patients with a recent MI but without diabetes could also provide important insights on the disease processes, but such a dataset was not available to us. Sixth, left ventricular ejection fraction could provide important information but it is not available in the dataset. Lastly, only baseline (randomisation) Olink® samples were available and a biomarker follow-up determination could not be performed.

Conclusions

The addition of multi-proteomic biomarkers to a clinical model in this population with diabetes and a recent MI, showed that ischemia and myocardial stretch are major outcome drivers in this population, and allowed a better risk prediction and event reclassification, potentially helping for better risk assessment and targeted treatment decisions.

Availability of Data and Materials

The data and materials may be available upon reasonable request.

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Disclosures

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JPF wrote the manuscript, performed the statistical analysis and made critical revisions; AS contributed to discussion and reviewed/edited the manuscript; CM contributed to discussion and reviewed/edited the manuscript including the revision of the statistical methods and analysis; GB reviewed/edited the manuscript; PR reviewed/edited the manuscript; WBW reviewed/edited the manuscript; FZ contributed to discussion and reviewed/edited the manuscript.

Dr. João Pedro Ferreira is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Characteristics of the study population with vs. without a primary outcome event

Variable	Primary outcome		p-value
	No	Yes	
N.	4541	590	
Age, years	60.6 ± 9.9	63.8 ± 9.8	<0.001
Male sex	3107 (68.4%)	379 (64.2%)	0.041
Diabetes duration, years	6.8 (2.5, 13.3)	10.1 (4.7, 16.3)	<0.001
Body mass index, Kg/m ²	29.5 ± 5.5	29.7 ± 6.0	0.35
Smoking	634 (14.0%)	70 (11.9%)	0.16
Hypertension	3727 (82.1%)	543 (92.0%)	<0.001
Prior myocardial infarction	3962 (87.2%)	549 (93.1%)	<0.001
Prior heart failure diagnosis	1231 (27.1%)	241 (40.8%)	<0.001
Prior stroke	279 (6.1%)	85 (14.4%)	<0.001
Peripheral artery disease	389 (8.6%)	99 (16.8%)	<0.001
Atrial fibrillation	285 (6.3%)	75 (12.7%)	<0.001
Heart rate, bpm	71.3 ± 10.7	72.1 ± 11.6	0.11
Systolic blood pressure, mmHg	128.7 ± 16.5	132.3 ± 17.5	<0.001
Diastolic blood pressure, mmHg	76.5 ± 9.6	75.8 ± 10.6	0.12
Insulin use	1312 (28.9%)	229 (38.8%)	<0.001
eGFR, ml/min/1.73 m ²	72.0 ± 21.0	62.4 ± 22.8	<0.001
Total cholesterol, mg/dL	153.1 ± 43.0	164.2 ± 48.2	<0.001
LDL cholesterol, mg/dL	77.5 ± 33.9	87.3 ± 39.3	<0.001
HDL cholesterol, mg/dL	43.1 ± 10.4	43.4 ± 11.4	0.53
Triglycerides, md/dL	164.2 ± 105.6	167.5 ± 93.2	0.48

Legend: eGFR, estimated glomerular filtration rate.

The primary outcome was a composite of cardiovascular death, non-fatal myocardial infarction or non-fatal stroke.

Table 2. Clinical model for event prediction

Variables	HR	95%CI	z-score	p-value
eGFR (per ml/min/1.73m ²)	0.99	0.98 : 0.99	-5.7	<0.001
Stroke	1.75	1.40 : 2.20	4.9	<0.001
Previous HFH	1.49	1.26 : 1.75	4.7	<0.001
Myocardial infarction	1.93	1.41 : 2.63	4.1	<0.001
Diabetes duration (per year)	1.02	1.01 : 1.03	4.0	<0.001
Hypertension	1.70	1.25 : 2.31	3.4	0.001
SBP	1.01	1.00 : 1.01	3.3	0.001
Atrial fibrillation	1.51	1.18 : 1.92	3.3	0.001
Age (per year)	1.01	1.00 : 1.02	1.9	0.058
Smoking	1.14	0.89 : 1.47	1.1	0.29
Female	0.96	0.81 : 1.13	-0.5	0.61
Alogliptin	0.97	0.83 : 1.14	-0.4	0.71
Statin	0.99	0.76 : 1.31	-0.1	0.96

Legend: HFH, heart failure hospitalization; dur., duration; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure.

Variables ordered by their prognostic “importance” (z-score) for the primary outcome of cardiovascular death, MI or stroke.

C-index =0.68 for the primary outcome of cardiovascular (CV) death, MI, or stroke; =0.75 for CV death; =0.74 for all-cause death; =0.76 for CV death or HFH; =0.79 for HFH; =0.66 for MI; =0.70 for stroke.

Goodness of fit test p-value >0.10 for all the studied outcomes i.e. good model calibration (see also the Supplemental Figure 1).

Table 3. Multivariable final biomarker selection for each outcome (on top of the clinical model)

Biomarker	HR	95%CI		z-score	p-value
<i>Primary outcome (CV death, MI, stroke): C-index =0.73</i>					
TROPONIN	1.18	1.11	1.26	5.2	<0.001
BNP	1.11	1.06	1.17	4.1	<0.001
TRAILR2	1.25	1.06	1.48	2.6	0.008
VEGFD	1.33	1.06	1.68	2.4	0.015
FGF23	1.10	1.02	1.20	2.4	0.015
MMP7	1.10	1.02	1.19	2.4	0.015
<i>CV death: C-index =0.82</i>					
BNP	1.29	1.19	1.41	5.9	<0.001
TROPONIN	1.23	1.12	1.35	4.2	<0.001
TRAILR2	1.45	1.11	1.88	2.8	0.006
CEACAM8	1.23	1.05	1.44	2.5	0.012
VEGFD	1.60	1.08	2.37	2.3	0.019
<i>All-cause death: C-index =0.80</i>					
BNP	1.26	1.18	1.35	6.6	<0.001
TROPONIN	1.20	1.10	1.30	4.3	<0.001
TRAILR2	1.50	1.21	1.87	3.7	<0.001
CEACAM8	1.22	1.05	1.41	2.7	0.008
ADAMTS13	0.56	0.34	0.94	-2.2	0.028
TF	1.48	1.01	2.16	2.0	0.044
<i>CV death or HFH: C-index =0.83</i>					
BNP	1.25	1.17	1.34	6.7	<0.001
TROPONIN	1.19	1.10	1.28	4.5	<0.001
TRAILR2	1.51	1.24	1.85	4.1	<0.001
VEGFD	1.88	1.38	2.56	4.0	<0.001

GAL9	1.46	1.04	2.05	2.2	0.031
HFH: C-index =0.86					
BNP	1.16	1.05	1.28	3.4	0.004
GAL9	1.91	1.16	3.16	3.3	0.011
VEGFD	1.94	1.07	3.51	3.1	0.029
FGF23	1.16	1.03	1.31	3.0	0.014
TROPONIN	1.16	1.04	1.30	3.0	0.010
SCF	0.69	0.52	0.93	-2.3	0.015
GIF	0.90	0.82	0.99	-2.1	0.032
MI: C-index =0.69					
TROPONIN	1.16	1.08	1.25	4.4	<0.001
FGF23	1.16	1.06	1.28	3.2	0.001
AMBP	2.08	1.26	3.44	2.8	0.004
MMP7	1.14	1.03	1.26	2.6	0.011
Stroke: C-index =0.73					
TROPONIN	1.35	1.16	1.57	3.3	<0.001
ADAMTS13	0.22	0.10	0.51	2.5	<0.001

Legend: BNP, brain natriuretic peptide; TRAILR2, trail receptor 2 (or death receptor 5); VEGFD, vascular endothelial growth factor D; FGF23, fibroblast growth factor 23; AMBP, alpha-1-microglobulin/bikunin precursor; MMP7, matrix-metalloproteinase 7; CEACAM8, carcinoembryonic antigen related cell adhesion molecule 8; ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; TF, transferrin; GAL9, galectin 9; SCF, stem cell factor; GIF, gastric intrinsic factor.

Light blue cells, biomarkers that add prognostic information to the multivariable model based on C-index and BIC improvement by the sequential addition (z-score based) of each individual biomarker to the clinical model, then the second biomarker was added to the clinical model plus the first biomarker, and henceforth (please see also the figure 1 and the supplemental figure 2).

All biomarkers are expressed in normalized NPX units, except Troponin expressed in Log2 units.

Table 4. Net Reclassification Improvement for the study outcomes

Outcome	NRI% (95%CI)	p-value (global)
Primary outcome: Clinical model vs. Clinical+Troponin+BNP		
cNRI event	10% (4-16)	<0.001
cNRI non-event	26% (25-32)	
cNRI global	37% (31-48)	
CV death: Clinical model vs. Clinical+BNP+Troponin		
cNRI event	29% (21-35)	<0.001
cNRI non-event	35% (31-37)	
cNRI global	64% (52-70)	
All-cause death: Clinical model vs. Clinical+BNP+Troponin+TRAILR2		
cNRI event	28% (20-31)	<0.001
cNRI non-event	33% (29-36)	
cNRI global	60% (49-65)	
CV death/HFH: Clinical model vs. Clinical+BNP+Troponin+TRAILR2		
cNRI event	26% (24-34)	<0.001
cNRI non-event	33% (31-36)	
cNRI global	59% (57-70)	
HFH: Clinical model vs. Clinical+BNP+Gal9		
cNRI event	27% (16-37)	<0.001
cNRI non-event	26% (22-32)	
cNRI global	53% (38-68)	
MI: Clinical model vs. Clinical+Troponin+FGF23+AMBP		
cNRI event	2% (1-10%)	<0.001
cNRI non-event	21% (16-25)	
cNRI global	23% (21-33)	
Stroke: Clinical model vs. Clinical+Troponin		

cNRI event	8% (-26-18)	<0.001
cNRI non-event	32% (28-38)	
cNRI global	23% (2-50)	

Legend: cNRI, continuous net reclassification improvement; BNP, brain natriuretic peptide;

TRAILR2, trail receptor 2 (or death receptor 5); Gal9, galectin 9; FGF23, fibroblast growth factor 23;

AMBP, alpha-1-microglobulin/bikunin precursor.

For example, a cNRI of 10% for primary outcome events means that 10% of patients who had a primary outcome event and were classified incorrectly (i.e. as no event) by the clinical model have been reclassified correctly (i.e. as events) under the model that also includes troponin and BNP.

Table 5. Events and event-rates by the patients` risk profile

Outcome / Quintiles of risk	Clinical model		Clinical + Biomarker(s) model*	
	Events n. (%)	Event-rate (100py)	Events n. (%)	Event-rate (100py)
Primary outcome				
Q1	43 (4.0)	2.6 (1.9-3.4)	33 (3.3)	2.0 (1.5-2.9)
Q2	74 (6.9)	4.4 (3.5-5.5)	56 (5.5)	3.5 (2.7-4.6)
Q3	98 (9.2)	6.1 (5.0-7.4)	94 (9.3)	6.1 (5.0-7.5)
Q4	143 (13.4)	8.9 (7.5-10.5)	140 (13.8)	9.3 (7.9-11.0)
Q5	261 (24.4)	18.6 (16.4-21.0)	261 (25.8)	19.9 (17.6-22.4)
Cardiovascular death				
Q1	7 (0.7)	0.4 (0.2-0.9)	4 (0.4)	0.2 (0.1-0.6)
Q2	13 (1.2)	0.7 (0.4-1.3)	8 (0.8)	0.5 (0.3-1.0)
Q3	40 (3.7)	2.3 (1.7-3.2)	23 (2.3)	1.4 (1.0-2.2)
Q4	56 (5.2)	3.3 (2.5-4.3)	39 (3.9)	2.4 (1.8-3.3)
Q5	124 (11.6)	7.9 (6.6-9.4)	148 (14.6)	10.1 (8.6-11.9)
All-cause death				
Q1	15 (1.4)	0.9 (0.5-1.5)	9 (0.9)	0.5 (0.3-1.0)
Q2	18 (1.7)	1.0 (0.7-1.6)	11 (1.1)	0.7 (0.4-1.2)
Q3	46 (4.3)	2.7 (2.0-3.6)	40 (4.0)	2.5 (1.9-3.4)
Q4	78 (7.3)	4.6 (3.7-5.7)	60 (5.9)	3.7 (2.9-4.8)
Q5	167 (15.6)	10.6 (9.1-12.4)	178 (17.6)	12.2 (10.5-14.1)
Cardiovascular death or HF hospitalization				
Q1	17 (1.6)	1.0 (0.6-1.6)	4 (0.4)	0.2 (0.1-0.6)
Q2	23 (2.2)	1.3 (0.9-2.0)	11 (1.1)	0.7 (0.4-1.2)
Q3	52 (4.9)	3.1 (2.3-4.0)	41 (4.1)	2.6 (1.9-3.5)
Q4	102 (9.5)	6.3 (5.2-7.7)	71 (7.0)	4.6 (3.6-5.7)
Q5	204 (19.1)	13.7 (12.0-15.7)	241 (23.8)	18.1 (15.9-20.5)

HF hospitalization				
Q1	5	0.3 (0.1-0.7)	1 (0.1)	0.1 (0.0-0.4)
Q2	13	0.7 (0.4-1.3)	5 (0.5)	0.3 (0.1-0.7)
Q3	24	1.4 (0.9-2.1)	15 (1.5)	0.9 (0.6-1.5)
Q4	32	2.0 (1.4-2.8)	33 (3.2)	2.1 (1.5-2.9)
Q5	119	8.0 (6.7-9.5)	129 (12.6)	9.5 (8.0-11.3)
Myocardial infarction				
Q1	25 (2.3)	1.5 (1.0-2.2)	21 (2.1)	1.3 (0.8-2.0)
Q2	56 (5.2)	3.3 (2.5-4.3)	43 (4.2)	2.7 (2.0-3.6)
Q3	63 (5.9)	3.9 (3.0-5.0)	62 (6.1)	4.0 (3.1-5.1)
Q4	81 (7.6)	5.1 (4.1-6.3)	73 (7.2)	4.9 (3.9-6.1)
Q5	135 (12.6)	9.3 (7.9-11.0)	144 (14.2)	11.0 (9.3-12.9)
Stroke				
Q1	5	0.3 (0.1-0.7)	3 (0.3)	0.2 (0.1-0.6)
Q2	8	0.5 (0.2-0.9)	8 (0.8)	0.5 (0.2-1.0)
Q3	8	0.5 (0.2-0.9)	5 (0.5)	0.3 (0.1-0.7)
Q4	8	0.5 (0.2-0.9)	11 (1.1)	0.7 (0.4-1.2)
Q5	32	2.0 (1.4-2.8)	34 (3.3)	2.2 (1.6-3.1)

Legend: Q, quintile of risk; HF, heart failure.

Each “risk model” was derived from the linear predictor (y_j) of each model (clinical or biomarker) for each outcome, where each dependent variable is multiplied by its β coefficient (e.g., $y_j = b_1 * x_{1j} + b_2 * x_{2j} + \dots + b_k * x_{kj}$) and then divided in quintiles with similar number of patients.

Each quintile of risk includes 1013 patients, total =5065.

Median follow-up time =1.6 (1.0-2.2) years.

*The biomarkers used for each outcome are the same as described in Table 2 and Table 3 (Primary outcome: Clinical model vs. Clinical+Troponin+BNP; CV death: Clinical model vs. Clinical+BNP+Troponin; All-cause death: Clinical model vs. Clinical+BNP+Troponin+TRAILR2;

CV death/HFH: Clinical model vs. Clinical+BNP+Troponin+TRAILR2; HFH: Clinical model vs. Clinical+BNP+Gal9; MI: Clinical model vs. Clinical+Troponin+FGF23+AMBP; Stroke: Clinical model vs. Clinical+Troponin) and the same clinical variables as described in the Table 1 (Previous HFH, Diabetes duration (per year), Myocardial infarction, eGFR (per ml/min/1.73m2), Stroke, SBP, Hypertension, Atrial fibrillation, Age (per year), Smoking, Female, Alogliptin, Statin).

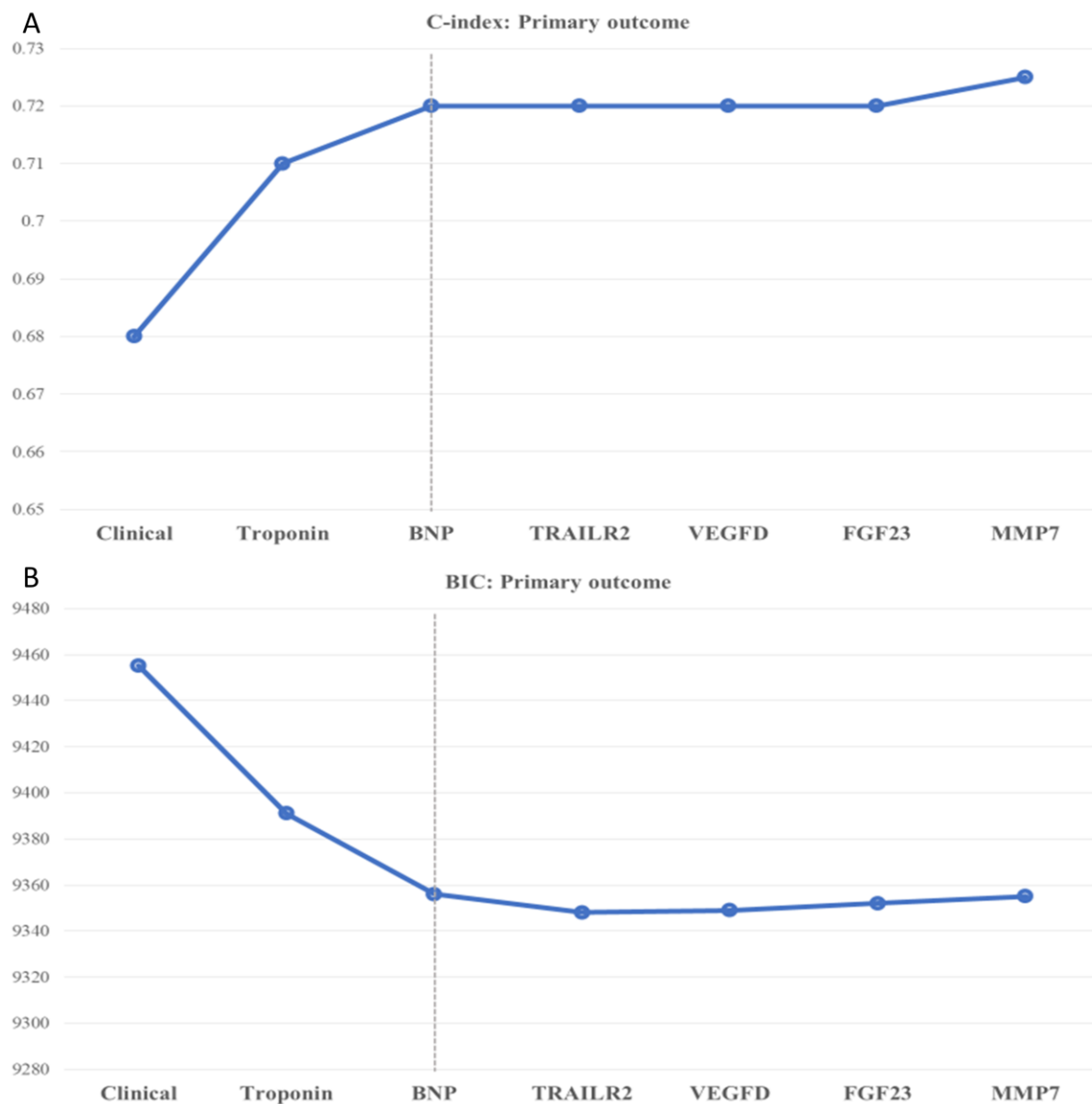


Figure 1. Multivariable c-index and Bayesian Information Criteria (BIC) for the primary outcome

(A) C-index

(B) BIC

Legend: BNP, brain natriuretic peptide; TRAILR2, trail receptor 2 (or death receptor 5); VEGFD, vascular endothelial growth factor D; FGF23, fibroblast growth factor 23; MMP7, matrix-metalloproteinase 7. No added value to the model after BNP (dashed line) i.e. c-index and BIC p-value >0.05. Please see also the Supplemental Figure 2 for the full results.

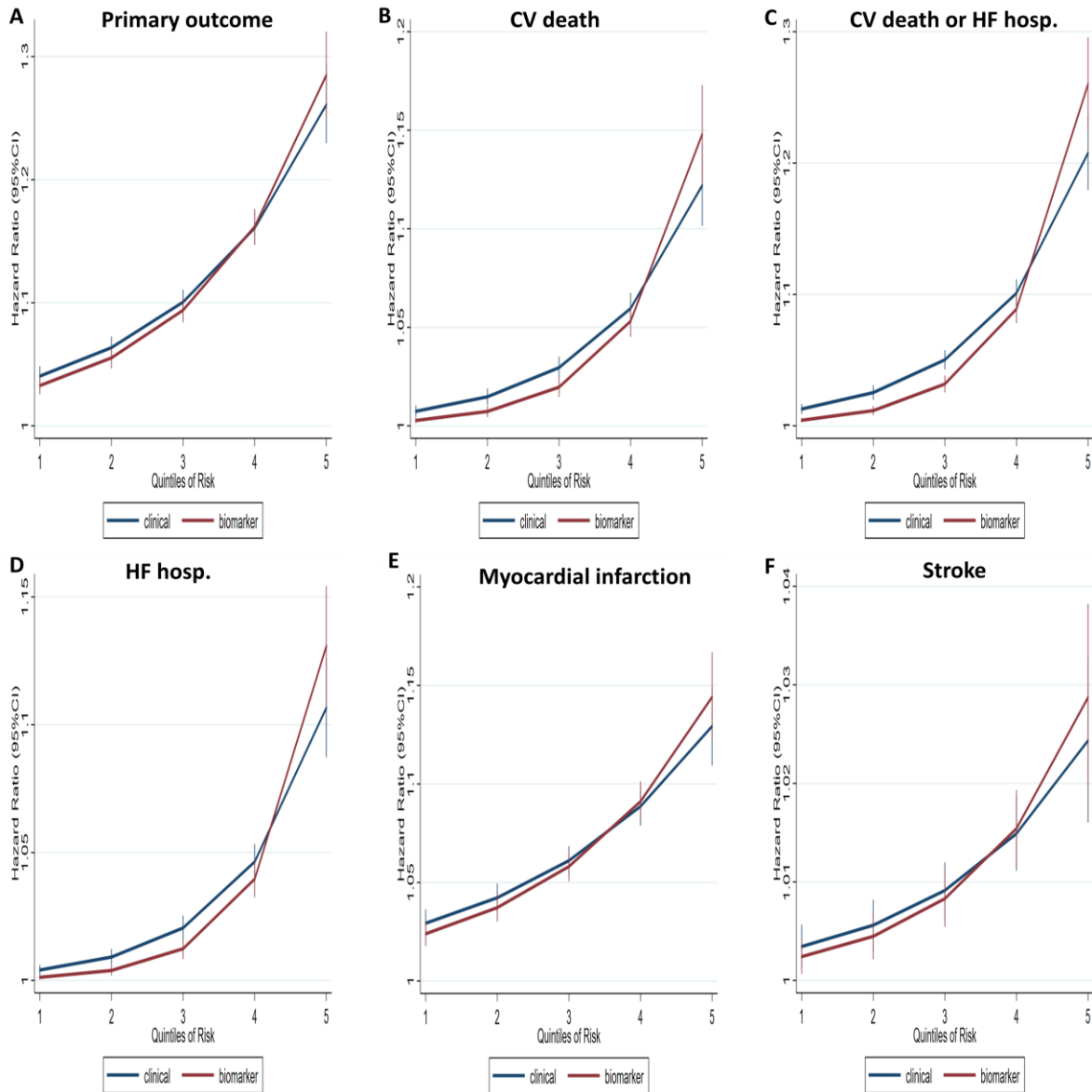


Figure 2. Comparison of the clinical model with the clinical plus biomarker model

(A) Primary outcome

(B) Cardiovascular death

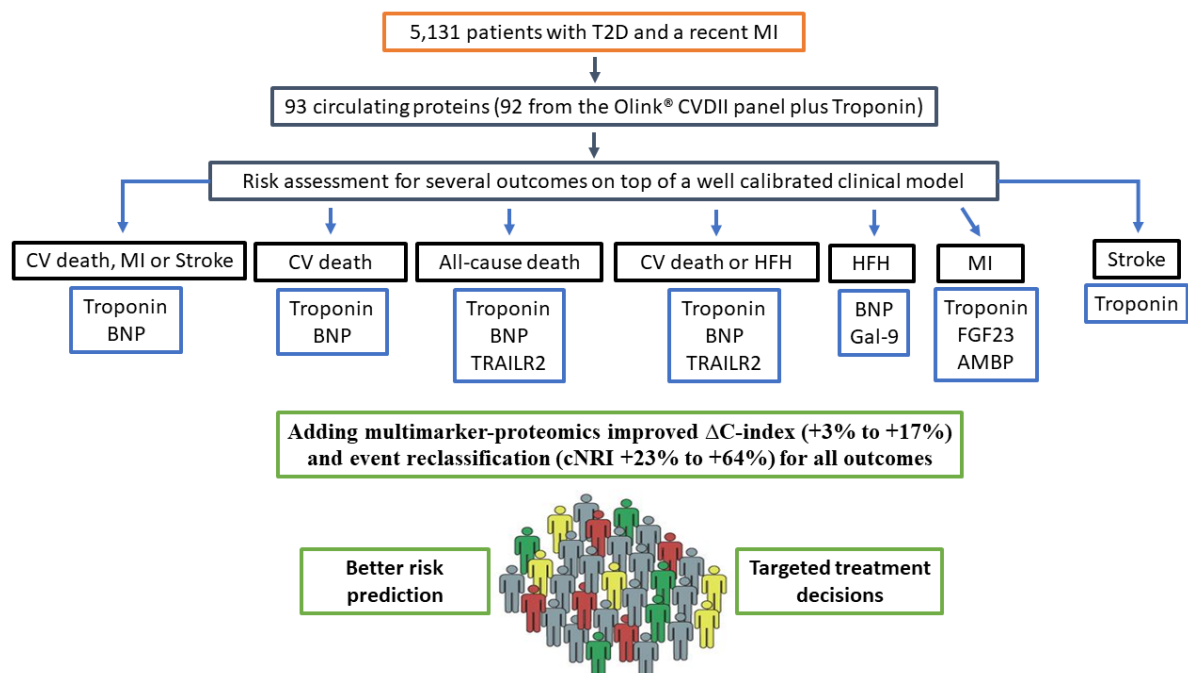
(C) Cardiovascular death or Heart Failure Hospitalization

(D) Heart Failure Hospitalization

(E) Myocardial Infarction

(F) Stroke

Legend: CI, confidence interval. The clinical plus biomarker model outperforms the clinical model by reclassifying both patients at low risk as “true” low risk and patients at high event risk as “true” high risk. The primary outcome was a composite of cardiovascular death, myocardial infarction or stroke.



Graphical abstract. Multi-Proteomic approach to predict specific cardiovascular events in patients with diabetes and myocardial infarction: findings from the EXAMINE trial

Legend: T2D, type 2 diabetes; MI, myocardial infarction; CV, cardiovascular; HFH, heart failure hospitalization; Δ , delta; cNRI, continuous net reclassification index; BNP, brain natriuretic peptide; TRAILR2, trail receptor 2 (or death receptor 5); Gal-9, galectin-9; FGF23, fibroblast growth factor 23.