Urinary Proteome and Systolic Blood Pressure as Predictors of 5-Year Cardiovascular and Cardiac Outcomes in a General Population

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Abstract—In a previous cross-sectional study, we identified a multidimensional urinary classifier (HF1), which was associated with left ventricular dysfunction. We investigated whether HF1 predicts cardiovascular end points over and beyond traditional risk factors. In 791 randomly recruited Flemish (mean age, 51.2 years; 50.6% women), we quantified HF1 by capillary electrophoresis coupled with mass spectrometry. In addition, we measured cardiovascular risk factors. HF1 averaged −0.97 U (range, −3.26 to 2.60). Over 6.1 years (median), 35 participants died and 63, 45, and 22 experienced fatal or nonfatal cardiovascular, cardiac, or coronary events, respectively. The incidence of fatal combined with nonfatal cardiovascular and cardiac end points, standardized for sex and age, increased across thirds of the HF1 distribution ($P \leq 0.014$), whereas trends for all-cause mortality and coronary events were nonsignificant ($P \geq 0.10$). The multivariable-adjusted hazard ratios (+1-SD) were 1.30 (95% confidence interval, 1.03–1.65; $P=0.029$) and 1.39 (1.06–1.84; $P=0.018$) for cardiovascular and cardiac events in relation to HF1. For systolic pressure, the corresponding estimates were 0.97 (0.74–1.28; $P=0.85$) and 0.93 (0.67–1.29; $P=0.66$), respectively. The HF1 upper thresholds optimized by maximizing Younden's index were −0.50 and −0.36 U for cardiovascular and cardiac end points, respectively. Prognostic accuracy significantly ($P \leq 0.006$) improved by adding HF1 to Cox models already including the other baseline predictors. Sensitivity analyses, from which we excluded 71 participants with previous cardiovascular disease, were confirmatory. In conclusion, over a 6-year period, the urinary proteome, but not systolic pressure, predicted cardiovascular and cardiac disease. **(*Hypertension*. 2015;66:52-60. DOI: 10.1161/HYPERTENSIONAHA.115.05296.)** • **Online Data Supplement**

Key Words: biomarker ■ cardiovascular disease ■ morbidity ■ mortality ■ proteomics

Recent publications, reviewed elsewhere,[1] proved the feasibility of developing multidimensional classifiers based on the urinary proteome that are associated with chronic kidney disease,[2] urothelial cell carcinoma,[3] prostate cancer,[4] or coronary heart disease.[5] However, these urinary proteomic markers were mainly established in selected patients matched with controls or in cohorts of diseased patients. In 2012, we identified in a preliminary case–control study 85 urinary peptides, mainly up- or downregulated collagen fragments, that discriminated 19 hypertensive patients with asymptomatic diastolic left ventricular dysfunction from 19 controls.[6] With adjustments applied for multiple testing, 3 urinary peptide biomarkers remained significant.[6] In a subsequent cross-sectional study, we demonstrated that left ventricular diastolic dysfunction was associated with this proteomic signature in a random population sample.[7] A study in the same population revealed that the urinary proteome predicted progression of chronic kidney disease from grade 2 or lower to stage 3 or higher.[8] In the light of our previous observations,[6–8] we investigated in a random population sample whether the urinary proteome predicts cardiovascular events over and beyond traditional risk factors.

Methods

Study Participants

The Ethics Committee of the University of Leuven approved the Flemish Study on Environment, Genes and Health Outcomes.[9,10]

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Recruitment started in 1985 and continued until 2004. The initial participation rate was 78.0%. The participants were repeatedly followed up.[9,10] From May 2005 until May 2010, we mailed an invitation letter to 1208 former participants for a follow-up examination. However, 153 were unavailable because they had died earlier (n=26), because they had been institutionalized or were too ill (n=27), or because they had moved out of the area (n=100). Of the remaining 1055 former participants, 828 renewed informed consent. The participation rate was therefore 78.5%. We excluded 37 participants from analysis because urine samples for urinary proteomics were unavailable (n=22) or because they were lost to follow-up (n=15). Thus, the outcome cohort included 791 participants (for details, see Figure S1 in the online-only Data Supplement).

Assessment of Outcome

At annual intervals, we ascertained vital status through 6 October 2014 via the Belgian Population Registry in Brussels, Belgium. We obtained the International Classification of Disease codes for the immediate and underlying causes of death from the Flemish Registry of Death Certificates. For all 791 participants, we collected information on the incidence of nonfatal events either via a follow-up visit at the examination center with repeat administration of the same standardized questionnaire as used at baseline (n=637) or via a structured telephone interview (n=119).

Coronary events included sudden death, myocardial infarction, acute coronary syndrome, new-onset angina pectoris, ischemic cardiomyopathy, and coronary revascularization. Cardiac events additionally included heart failure, pulmonary heart disease, new-onset atrial fibrillation, life-threatening arrhythmias, and high-degree atrioventricular block requiring pacemaker implantation. Cardiovascular events comprised cardiac end points, stroke, transient ischemic attack, aortic aneurysm, arterial embolism, and revascularization of peripheral arteries. To assess the symptoms associated with heart failure, we administered the London School of Hygiene questionnaires on cardiovascular and respiratory symptoms and dyspnea. Physicians ascertained the diseases reported on the death certificates or by the questionnaires and interviews against the medical records of general practitioners or hospitals. For all end points within a category, we censored participants from analysis after the occurrence of the first event.

Urinary Proteomics

To assess the urinary proteome-based HF1 classifier, 24-h urine samples were processed and analyzed using capillary electrophoresis coupled with mass spectrometry as described previously.[11] The online-only Data Supplement (pages 2–3) gives detailed information on the preparation and processing of the urine samples. The data (on abundance) of the predefined urinary peptide biomarkers were combined into a single summary variable, using the support-vector machine–based MosaCluster software, version 1.7.0. In the present study, we used the previously published multidimensional classifier HF1, which is associated with decreased left ventricular function and which combines information from 85 peptide fragments identified in 19 patients with diastolic LV dysfunction and 19 controls.[6] How support-vector machine modeling establishes the HF1 classifier is described in the online-only Data Supplement (pages 3–4). In addition, the online-only Data Supplement provides information on the peptides making up HF1 (Table S1) and sequenced peptides with known amino-acid sequence (Table S2).

Other Measurements

Blood pressure was the average of 5 consecutive auscultatory readings obtained according to European guidelines with a standard mercury sphygmomanometer with the participant in the seated position for at least 10 minutes. As described elsewhere, we applied a stringent quality control program to the blood pressure measurements, looking for digit and number preference.[12,13] Hypertension was a blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic or use of antihypertensive drugs. Body mass index was weight in kilograms divided by height in meters squared. Venous blood samples were drawn for measurement of plasma glucose and serum total and high-density lipoprotein cholesterol, creatinine, and γ-glutamyltransferase (index of alcohol intake). Glomerular filtration rate was derived by the Chronic Kidney Disease Epidemiology Collaboration equation.[14] Diabetes mellitus was a self-reported diagnosis, a condition mentioned on the death certificate or in records held by general practitioners or hospitals, antidiabetic drug intake, a fasting plasma glucose level of at least 126 mg/dL, or a random blood glucose of at least 200 mg/dL.[15]

Statistical Analysis

For database management and statistical analysis, we used the SAS system, version 9.3 (SAS Institute Inc., Cary, NC). Means were compared using the large-sample z-test or ANOVA and proportions by Fisher exact test. Statistical significance was a 2-sided significance level of 0.05.

In exploratory analyses, we plotted incidence rates by tertiles of the urinary biomarker, while standardizing for sex and age group (<40 years; 40–60 years; >60 years) by the direct method. In categorical analyses with adjustments applied for sex and age, we plotted the cumulative incidence of cardiovascular and cardiac endpoints by thirds of the HF1 distribution. We used Cox regression to compute standardized hazard ratios, which for continuous variables express the risk associated with a 1-standard deviation (SD) increment in the urinary biomarker. All models accounted for clustering of failure times within pedigrees by fitting a shared frailty model. We checked the proportional hazard assumption using the Kolmogorov-type supremum test. The baseline characteristics considered as covariables in Cox regression were sex, age, body mass index, systolic blood pressure, fasting plasma glucose, serum creatinine, the total-to-high-density lipoprotein cholesterol ratio, γ-glutamyltransferase (as index of alcohol intake), smoking, history of cardiovascular disease, and treatment with diuretics (thiazides, loop diuretics, and aldosterone antagonists), β-blockers, inhibitors of the renin–angiotensin system (angiotensin-converting enzyme inhibitors or angiotensin type-1 receptor blockers), vasodilators (calcium channel blockers or α-blockers), or other antihypertensive drugs. We identified covariables to be retained in the analyses by a step-down procedure, removing the least significant covariable at each step until all P values of covariables were <0.15. We applied the generalized R^2 statistic to assess the contribution of HF1 and systolic blood pressure to risk over and beyond other risk factors. We calculated the multivariable-adjusted 5-year absolute risk of a cardiovascular or cardiac event across thirds of the distributions of HF1 and systolic blood pressure.

Finally, we determined optimal discrimination limits for HF1 by maximizing Younden's index (the maximum of sensitivity plus specificity minus 1). We then assessed the added prognostic accuracy of the optimized thresholds, using the integrated discrimination improvement (IDI) and the continuous net reclassification improvement (NRI).[16] IDI is the difference between the discrimination slopes of the basic model and the basic model extended with HF1. The discrimination slope is the difference in predicted probabilities (%) between subjects with and without end point. To calculate NRI,[16] we predicted in each subject the 5-year risk for an event from a Cox model with and without HF1. If $P_{(up/event)}$ is the percentage of subjects with events whose predicted probability is increased by adding HF1 to the model and if $P_{(up/nonevent)}$ is the percentage of subjects without events whose predicted probability is increased, then NRI equals $2\times[P_{(up/event)}-P_{(up/nonevent)}]$.

Results

Characteristics of Participants

Of 791 participants, 400 (50.6%) were women. All subjects were White Europeans. Mean values (SD) in all participants combined were 51.2 (15.7) years (interquartile range [IQR], 41.3–61.8 years) for age, 129.6 (17.7) and 79.7 (9.6) mm Hg

for systolic and diastolic blood pressure, 26.5 (4.3) kg/m^2 for body mass index, and 5.26 (0.97) mmol/L for total cholesterol. Of 3955 systolic and 3955 diastolic blood pressure readings, only 10 (0.13%) ended on an uneven number. With regards to number preferences, the 5 consecutive blood pressure readings included 3 identical systolic or diastolic values in 340 instances (4.3%) and 5 identical values in only a single participant.

Of all participants, 340 (43.0%) had hypertension, of whom 209 (61.5%) were on antihypertensive drug treatment, 27 (3.4%) had a history of diabetes mellitus, and 27 (3.4%) reported previous cardiovascular disease. The antihypertensive drug classes used were diuretics in 84 (10.6%) participants, β-blockers in 124 (15.7%), inhibitors of the renin–angiotensin system in 68 (8.6%), and vasodilators in 39 (4.9%). Among 209 treated patients, 122 (58.4%) were on monotherapy; 69 (33.0%) were taking 2 drug classes; and 18 (8.6%) were taking ≥3. Of 400 women and 391 men, 80 (20.0%) women and 80 (20.5%) men were smokers and 225 (56.3%) women and 323 (82.6%) men reported intake of alcohol. In smokers, median tobacco use was 13 cigarettes per day (IQR, 7–20 cigarettes per day). In drinkers, the median alcohol consumption was 8 g per day (IQR, 3–14 g per day).

Table 1 lists the characteristics of participants by thirds of the HF1 distribution (median; −1.03; IQR, −1.60 to −0.44). Age, body mass index, central obesity, blood pressure, serum creatinine, blood glucose and γ-glutamyltransferase, and the total-to- high-density lipoprotein cholesterol ratio all (P≤0.002) increased with higher HF1 category, whereas estimated glomerular filtration rate decreased (P<0.0001). The prevalence of hypertension, being treated for hypertension, having a history

Table 1. Baseline Characteristics of 791 Participants by Thirds of the HF1 Distribution

Characteristic	<−1.42	−1.42 to −0.68	≥−0.68	*P* Value
Number in category	264	263	264	
Number of subjects, %				
Women	138 (52.3)	133 (50.6)	129 (48.9)	0.74
Smokers	57 (21.6)	60 (22.8)	43 (16.3)	0.14
Drinking alcohol	185 (70.1)	191 (72.6)	172 (65.2)	0.17
Hypertension	80 (30.3)	94 (35.7)	166 (62.9)§	<0.0001
Antihypertensive treatment	37 (14.0)	50 (19.0)	122 (46.2)§	<0.0001
History of CVD	7 (2.7)	4 (1.5)	16 (6.1)†	0.011
History of diabetes mellitus	3 (1.1)	7 (2.7)	17 (6.4)*	0.003
Mean (SD) of characteristic				
Age, y	45.6 (15.3)	50.4 (14.3)‡	57.5 (15.0)§	<0.0001
Body mass index, kg/m^2	25.6 (3.8)	26.0 (4.0)	28.0 (4.8)§	<0.0001
Waist-to-hip ratio	0.85 (0.08)	0.87 (0.08)*	0.90 (0.08)§	<0.0001
Office blood pressure, mm Hg				
Systolic pressure	126.6 (17.3)	127.9 (16.7)	134.3 (18.2)§	<0.0001
Diastolic pressure	78.4 (9.6)	79.7 (9.5)	81.1 (9.6)	0.004
Mean arterial pressure	94.4 (10.7)	95.8 (10.5)	98.9 (10.7)‡	<0.0001
Heart rate, bpm	62.6 (8.6)	63.4 (9.3)	64.4 (11.1)	0.10
Biochemical data				
Serum creatinine, μmol/L	81.8 (13.3)	83.7 (13.1)	86.7 (19.8)*	0.002
eGFR, mL/min/1.73 m^2	83.9 (16.4)	80.2 (16.2)†	76.3 (16.2)†	<0.0001
Total cholesterol, mmol/L	5.13 (0.95)	5.32 (0.98)*	5.33 (0.98)	0.028
HDL cholesterol, mmol/L	1.45 (0.34)	1.45 (0.37)	1.37 (0.32)†	0.008
THR	3.70 (0.98)	3.84 (1.03)	4.06 (1.05)*	0.0002
Blood glucose, mmol/L	4.85 (0.56)	4.88 (0.58)	5.09 (1.07)†	0.0007
γ-GT, units/L	21 (11, 40)	24 (12, 57)*	26 (13, 55)	0.0005

CVD indicates cardiovascular disease; eGFR, estimated glomerular filtration rate calculated according to the Chronic Kidney Disease Epidemiology Collaboration equation formula, as described in reference 14; γ-GT, γ-glutamyltransferase; HDL, high-density lipoprotein; and THR, total-to-HDL cholesterol ratio. Office blood pressure was the average of 5 consecutive readings. Hypertension was an office blood pressure of ≥140 mm Hg systolic, or ≥90 mm Hg diastolic, or use of antihypertensive drugs. For γ-glutamyltransferase, values are geometric mean (interquartile range). Diabetes mellitus was a self-reported diagnosis, a condition mentioned on the death certificate or in records held by general practitioners or hospitals, antidiabetic drug intake, a fasting glucose level of at least 126 mg/dL, or a random blood glucose at least 200 mg/dL. *P* values denote the significance of the differences in prevalence rates or means across thirds of the HF1 distribution.

Significance of the difference with the adjacent lower third: *P≤0.05; †P≤0.01; ‡P≤0.001; §P≤0.0001.

of cardiovascular disease, or diabetes mellitus increased with HF1. The proportions of women, smokers, and average heart rate did not differ across HF1 categories ($P \geq 0.10$).

Incidence of Events

Median follow-up was 6.1 years (5th to 95th percentile, 3.7–7.4). During 4552 person-years of follow-up, 35 participants died, 11 of cardiovascular disease, and 63 experienced a fatal or nonfatal cardiovascular event (14.4 events per 1000 person-years). Fatal or nonfatal cardiac event occurred in 45 participants (10.1 events per 1000 person-years) and coronary events in 22 (4.9 events per 1000 person-years). Table S3 lists the cause-specific cardiovascular mortality and morbidity for the study cohort.

Risk Associated with HF1 Categories

Crude rates of cardiovascular, cardiac, and coronary events increased across thirds of the HF1 distribution (Table S4; $P \leq 0.0032$) with a similar trend for all-cause mortality ($P=0.057$). The incidence of fatal combined with nonfatal cardiovascular ($P=0.014$) and cardiac ($P=0.009$) end points, standardized for sex and age group, increased across thirds of the HF1 distribution (Figure 1), whereas the trends for all-cause mortality and coronary events did not reach significance ($P \geq 0.10$). For cardiovascular ($P=0.046$) and cardiac ($P=0.028$) events, the cumulative incidence ran a significantly higher course in the top compared with the low third of the HF1 distribution (Figure 2). These estimates were standardized to the means of the distributions of sex and age in the whole study population. Based on these results, the risk of cardiovascular and cardiac events was carried forward in multivariable-adjusted analyses.

Multivariable-Adjusted Risk Associated with the HF1 Biomarker

The step-down selection procedure identified sex, age, fasting blood sugar, smoking, and treatment with vasodilators as

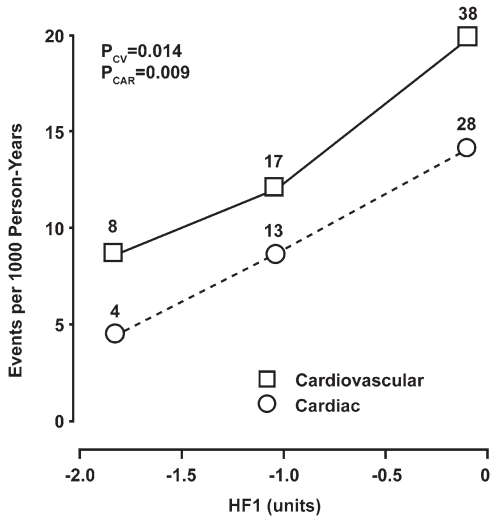

Figure 1. Incidence of fatal and nonfatal cardiovascular and cardiac events by tertiles of the distributions of the HF1 in 791 participants. Incidence rates were standardized for sex and age group (<40 years, 40–60 years, and >60 years) by the direct method. The number of end points contributing to the rates is shown. *P* values are for trend.

significant covariables of the risk of a cardiovascular or cardiac event (Table S5). Table 2 shows the multivariable-adjusted hazard ratios associated with a 1-SD increment in HF1. All Cox models complied with the proportional hazards assumption ($P \geq 0.056$). In all 791 participants, HF1 was a significant predictor of fatal and nonfatal cardiovascular ($P=0.029$) and cardiac events ($P=0.018$), whereas systolic blood pressure was not ($P=0.85$ and $P=0.66$, respectively). The generalized R^2 statistics for entering HF1 or systolic blood pressure (Table 2) as predictor in these models were 0.59 versus 0.01 for cardiovascular events and 0.69 versus 0.03 for cardiac events. Sensitivity analyses, from which we excluded 71 participants with previous cardiovascular disease (Table 2), or in all 791 participants with hypertension forced into the Cox models as additional covariable (P values for HF1, ≤ 0.042; data not shown) were confirmatory. Figure 3 shows the 5-year absolute risk of a cardiovascular or cardiac event in relation to systolic blood pressure (Figure 3A and 3B) at different levels of HF1 or in relation to HF1 (Figure 3C and 3D) at different levels of systolic blood pressure. The analyses were standardized to the average of the distributions in the whole study population (mean or ratio) of sex, age, fasting plasma glucose, smoking, and treatment with vasodilators. Figure 3 illustrates that HF1, being significantly related to outcomes ($P \leq 0.033$), was more informative than systolic pressure ($P \geq 0.54$) in predicting cardiovascular and cardiac events over a 5-year span.

Improvement of Prognostic Accuracy

By maximizing Younden's index (Table 3), we determined optimal thresholds for HF1 in predicting cardiovascular or cardiac events. Sensitivity of the optimized thresholds ranged from 59% to 86% for cardiovascular events and from 53% to 58% for cardiac events. Specificity ranged from 46% to 75% and from 80% to 81%, respectively. In all 791 participants (Table 4), both IDI and NRI reached significance for cardiovascular and cardiac events ($P \leq 0.006$). In 720 participants free of cardiovascular disease at entry (Table 4), IDI and NRI were significant for cardiovascular events ($P<0.0001$) and reached borderline significance for cardiac events ($P \leq 0.086$).

Discussion

The key findings of our study were as follows: (i) over a follow-up period of ≈5 years, the multidimensional urinary classifier HF1 predicted the incidence of fatal and nonfatal cardiovascular and cardiac events; (ii) after optimizing the HF1 thresholds, adding HF1 to Cox models, including baseline cardiovascular risk factors, significantly improved prognostic accuracy; (iii) HF1 was more informative than systolic blood pressure in the short-term prediction of cardiovascular and cardiac events. Our current findings are in line with a previous publication showing that in the same cohort, the early diastolic peak velocity of the mitral annulus (e') predicted the incidence of fatal combined with nonfatal cardiovascular events over a 5-year follow-up period.[17] Over a similar follow-up, mild and moderate-to-severe diastolic left ventricular dysfunction predicted all-cause mortality in the Olmsted County study with hazard ratios amounting to 8.3 and 10.2, respectively ($P<0.001$).[18] As previously demonstrated in

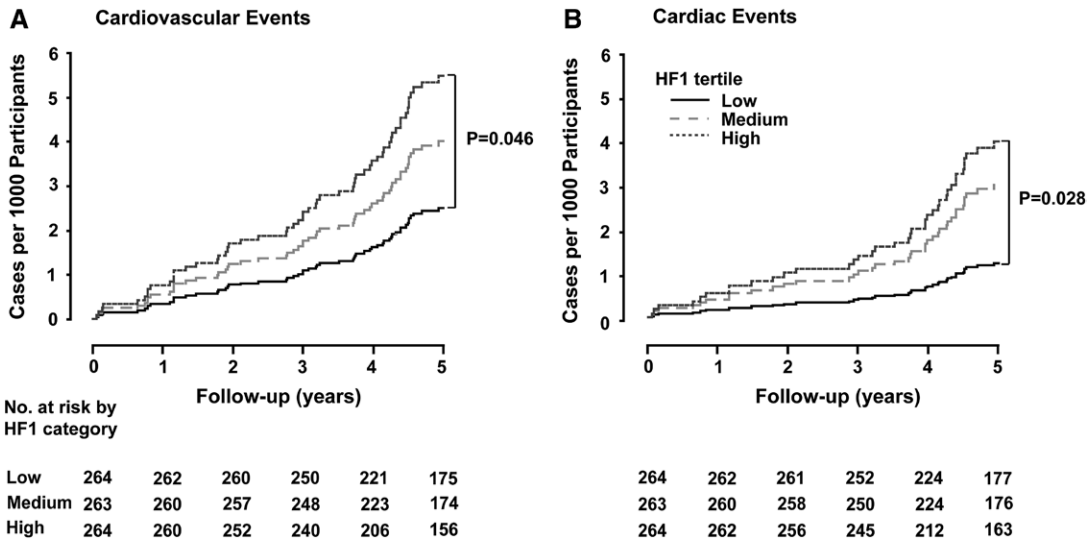

Figure 2. Cumulative incidence of cardiovascular (**A**) and cardiac end points (**B**) by thirds of the HF1 distribution. Estimates were standardized to the means of the distributions of sex and age in the whole study populations.

case–control[6] and proof-of-concept[7] studies, HF1 associates with diastolic left ventricular dysfunction.

Under physiological conditions, ≈70% of the urinary protein content originates from the kidney and the urinary tract, whereas the remaining 30% is derived from plasma.[19] We hypothesized that the latter fraction may be informative on cardiac and cardiovascular disease. The multidimensional urinary classifier HF1 combines information on 85 urinary peptides,[6] mainly upregulated and downregulated collagen fragments (Table S2). Patients with left ventricular dysfunction are characterized by a disturbed balance between collagen synthesis and degradation in the extracellular matrix of the myocardium and an increased interstitial deposition and cross-linking of type I collagen.[20] The cardiac extracellular matrix is predominantly composed of fibrillar collagen type I (85%) and type III (11%). Furthermore, small amounts of collagen types IV and V codistribute with collagen I. The prime location of collagen V is at the fibril core and is important in nucleating collagen I–containing fibrils.[21] The HF1 classifier also includes a downregulated peptide derived from the WW domain-binding protein 11 (Table S2) that remained statistically significant after adjustment for multiple testing.[6] WBP11 is an inhibitor of protein phosphatase-1.[22] In cardiomyocytes, protein phosphatase-1 plays a key role in calcium handling and relaxation via dephosphorylation of phospholamban.[23] In patients with heart failure, the activity of protein phosphatase-1 is enhanced, thereby delaying left ventricular relaxation. In line with this pathophysiological pathway, incident heart failure represented 33.3% and 46.7% of the cardiovascular and cardiac events, respectively. We also considered whether reducing the multidimensionality of classifiers, such as HF1, might be possible without losing information. However, we previously demonstrated that a higher number of peptides resulted in higher accuracy by reducing variability in the assessment of single components.[24] In an additional analysis of the current data, we therefore tested a classifier based only on the 3 peptide biomarkers remaining significant after adjustment for multiple testing.[6] As expected, this abridged classifier performed inferior to HF1 in diagnosing diastolic left ventricular dysfunction[7] and in the prediction of cardiovascular and cardiac events (data not shown).

In population studies with long-term follow-up, blood pressure is the main driver of cardiovascular risk.[25,26] In our current study, HF1 largely outperformed systolic blood pressure

Table 2. Multivariable-Adjusted Hazard Ratios Associated With HF1 and Systolic Blood Pressure

		HF1			Systolic Blood Pressure		
End Points	Events/At Risk	Hazard Ratio (95% CI)	*P* Value	R^2	Hazard Ratio (95% CI)	*P* Value	R^2
Cardiovascular events							
All participants	63/791	1.30 (1.03–1.65)	0.029	0.59	0.97 (0.74–1.28)	0.85	0.01
Free of CVD	42/720	1.39 (1.03–1.87)	0.034	0.59	1.00 (0.72–1.39)	0.98	<0.0001
Cardiac events							
All participants	45/791	1.39 (1.06–1.84)	0.018	0.69	0.93 (0.67–1.29)	0.66	0.03
Free of CVD	32/720	1.38 (0.98–1.95)	0.066	0.44	0.97 (0.66–1.40)	0.85	0.01

Hazard ratios express the risk per 1-SD increase in HF1 (0.92 U) and systolic blood pressure (17.7 mm Hg). Hazard ratios account for family clusters and were adjusted for baseline characteristics, including sex, age, fasting plasma glucose, smoking, and treatment with vasodilators (calcium-channel blockers [n=35] or α-blockers [n=4]). CVD indicates cardiovascular disease at entry. R^2 is expressed in percent.

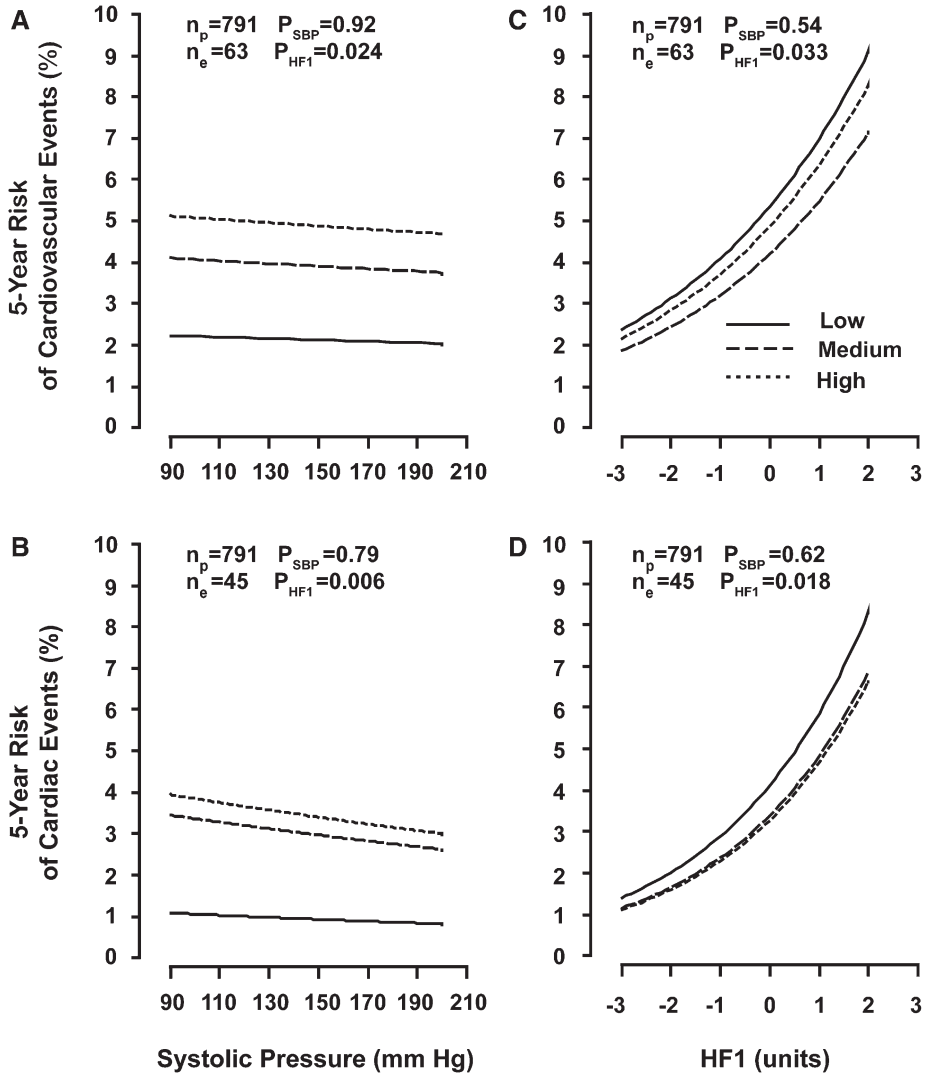

Figure 3. Five-year absolute risk of cardiovascular and cardiac events in relation to systolic blood pressure (**A**, **B**) at different categories of HF1 and in relation to HF1 (**C**, **D**) at different levels of systolic blood pressure. The analyses were standardized to the average of the distributions in the whole study population (mean or ratio) of sex, age, fasting plasma glucose, smoking, treatment with vasodilators (calcium-channel blockers or α-blockers). In **A** and **B**, the risk functions span the 5th to 95th percentile interval of systolic blood pressure and correspond to the low, medium, and high categories of HF1. In **C** and **D**, the risk functions span the 5th to 95th percentile intervals of HF1 and correspond to the low (<33th percentile), medium (33–66th percentile), and high (>66th percentile) categories of systolic blood pressure. *P* values are for the independent effect of HF1 (P_{HF1}) and systolic blood pressure (p_{SBP}). n_p and n_e indicate the number of participants at risk and the number of events.

in the prediction of cardiovascular and cardiac complications. These observations cannot be attributed to the quality of our blood pressure measurements, as exemplified by the absence of terminal digit and number preference. Several investigators have reported that single blood pressure measurements predicted the risk of cardiovascular disease over a follow-up

Table 3. Optimized HF1 Thresholds in the Prediction of Cardiovascular and Cardiac Events

End Points	Optimal Discrimination Limit	Hazard Ratio (95% CI)	Sensitivity, %	Specificity, %	Positive Predictive Value, %	Negative Predictive Value, %	Misclassification Rate, %
Cardiovascular events							
All participants (n=791)	–0.50	3.98 (2.41–6.57)	59	75	17	96	26
Free of CVD (n=720)	–1.22	4.84 (2.04–11.5)	86	46	9	98	52
Cardiac events							
All participants (n=791)	–0.36	5.01 (2.77–9.05)	58	80	15	97	22
Free of CVD (n=720)	–0.36	4.58 (2.29–9.18)	53	81	12	97	20

The optimal discrimination limit was obtained by maximizing Younden's index (the maximum of sensitivity plus specificity minus 1). Hazard ratios (95% confidence interval) present the risk associated with exceeding the biomarker threshold. The hazard ratios were significant ($P \leq 0.0003$).

Table 4. Integrated Discrimination Improvement and Net Reclassification Improvement by Adding HF1 to the Basic Model Including Covariables

	Integrated Discrimination Improvement			Net Reclassification Improvement		
End Points	IDI, %	CI, %	*P* Value	NRI, %	CI, %	*P* Value
Cardiovascular events						
All participants (n=791)	1.89	0.54–3.24	0.006	49.9	24.6–75.2	0.0001
Free of CVD (n=720)	2.13	1.33–2.93	<0.0001	63.8	41.3–86.2	<0.0001
Cardiac events						
All participants (n=791)	2.00	0.60–3.40	0.005	46.1	16.5–75.8	0.002
Free of CVD (n=720)	1.34	0.61–2.73	0.061	31.0	–4.37 to 66.3	0.086

The basic reference models included as covariables sex, age, fasting plasma glucose, smoking, and treatment with vasodilators. The IDI is the difference between the discrimination slopes of basic models and basic models extended with HF1. The discrimination slope is the difference in predicted probabilities (%) between cases and controls. Controls are participants without incident cardiovascular or cardiac disease. The NRI is the sum of the net percentages of subjects reclassified correctly in cases and controls. CI indicates confidence interval; IDI, integrated discrimination improvement; and NRI, net reclassification improvement.

period of 15 to 50 years, but also that the impact of blood pressure diminished with increasing duration of follow-up.[27–34] The strongest evidence in this regards comes from the Framingham Heart Study.[34] Vasan and coworkers used sex- and age-specific multivariable Cox regression to evaluate the association of current blood pressure (at baseline), recent antecedent blood pressure (average of readings for all available examinations 1–10 years before baseline), and remote antecedent blood pressure (average for all available examinations 11–20 years before baseline) with the 10-year risk of new-onset cardiovascular disease in 2313 Framingham Study participants. During follow-up, cardiovascular disease occurred in 899 participants. In multivariable-adjusted models, recent and remote antecedent blood pressure predicted the risk of cardiovascular disease incrementally over current blood pressure. This observation was consistent in multiple subgroups: women (n=1403) and men (n=910) and 3 age strata (60–69, 70–79, and ≥80 years).[34] Explanations offered by the Framingham investigators were that antecedent blood pressure is a forerunner of cardiovascular target organ damage, which is on the path to hard cardiovascular complications, and that the relation between cardiovascular risk and blood pressure might be weakened by the initiation of antihypertensive drug treatment.[34] Our current findings remained consistent after exclusion of participants with a history of cardiovascular disease. Similarly, estimates of the hazard ratios remained confirmatory if we forced hypertension as covariable in the Cox models or if we limited the analyses to participants untreated for hypertension at baseline (data not shown).

Use of vasodilators, calcium-channel blockers or α-blockers, at baseline was among the significant predictors of cardiovascular or cardiac events, for which our analyses were adjusted. Although resulting from an observational study, our findings are not surprising in view of the results of the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT).[35–37] In ALLHAT, use of doxazosin or amlodipine, compared with chlorthalidone was associated with an increased risk of combined cardiovascular disease. This secondary end point was driven by congestive heart failure[35–37] and coronary revascularization,[35–37] and for doxazosin also by hospitalized or treated angina pectoris.[37] Diuretics, β-blockers, angiotensin-converting enzyme inhibitors, and angiotensin type-1 receptor blockers are drug classes used in the primary and secondary prevention of cardiovascular complications, in particular heart failure, but in our current study, use of these drug classes at baseline did not predict cardiovascular or cardiac complications.

To our knowledge, there is no other population-based study that explored the value of the urinary proteome in predicting cardiovascular or cardiac complications. Nevertheless, our study must be interpreted within the context of its limitations. First, the sample size and number of events were substantially smaller than, for instance, in the Framingham Heart Study.[34] This might explain why we could not show any significant prognostic value of HF1 in relation to the incidence of mortality or coronary events. Second, our current study with a median follow-up of 6.1 years does not allow to compare the prognostic value of HF1 and blood pressure over a longer follow-up. Third, we did not assess the incremental predictive value of HF1 with other biomarkers of risk, such as high-sensitivity C-reactive protein[38] or high-sensitivity troponin T.[39]

Perspectives

Our current study demonstrated that the urinary proteome, but not systolic pressure, predicts incident cardiovascular and cardiac disease over a 5-year period and thereby confirmed the diagnostic utility of the urinary proteomic signature.[6–8] Further prospective studies should underpin its use in clinical practice compared with other biomarkers over varying intervals of follow-up in relation to different end points. A previous publication in the same cohort demonstrated that the early diastolic peak velocity of the mitral annulus (e') predicts the incidence of fatal combined with nonfatal cardiovascular events over a 5-year follow-up period.[17] When we introduced both HF1 and e' in multivariable-adjusted models (62 cardiovascular and 44 cardiac events in 776 participants who underwent an assessment of their diastolic left ventricular function; data not shown), both were predictive, albeit with borderline *P* values ($0.020 \leq P \leq 0.097$), reflecting complementarity of information.

However, for screening purposes, HF1 is less costly and labor-intensive than echocardiography and therefore preferable. Having the urinary proteome confirmed as a short-term predictor of adverse outcomes might allow the timely initiation of preventive or therapeutic measures. On the other hand, quality-of-life of patients confronted with a prognostically adverse urinary proteomic signature and cost-effectiveness remain issues of concern.

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Disclosures

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Novelty and Significance

What Is New?

- In a previous cross-sectional study, we identified a multidimensional urinary peptide-based classifier (HF1), which was associated with left ventricular dysfunction. In randomly recruited Flemish people, we investigated whether HF1 predicts cardiovascular end points over and beyond traditional risk factors.

What Is Relevant?

- Over a 5 year follow-up period, the standardized and multivariable-adjusted hazard ratios relating cardiovascular (n=63) and cardiac (n=45) events with HF1 were 1.30 and 1.39 ($P \leq 0.029$), respectively, whereas those with systolic pressure were around unity ($P \geq 0.66$).
- The diagnostic improvement obtained by adding optimized HF1 thresholds to Cox models including classical cardiovascular risk factors, including systolic blood pressure, was significant ($P \leq 0.006$).

Summary

Over ≈5 years of follow-up, the urinary proteome, but not systolic pressure, predicted incident cardiovascular disease. Further prospective studies should confirm its diagnostic utility compared with other biomarkers over varying intervals of follow-up in relation to different end points. Having the urinary proteome confirmed as a short-term predictor of adverse outcomes might allow the timely initiation of preventive or therapeutic measures.

HYPERTENSION

Supplementary Appendix

This appendix is part of the original submission and has been peer reviewed.
Supplement to *The Urinary Proteome and Systolic Blood Pressure as Predictors of 5-Year Cardiovascular and Cardiac Outcomes in a General Population*. Hypertension 2015; published online 12 May 2015 (DOI: 10.1161/HYPERTENSIONAHA.115.05296).

Expanded Methods

Urinary Proteomics

Aliquots of urine were stored at -80 °C. Urine (0.7 mL) was thawed immediately before analysis and diluted with 0.7 mL of 2 M urea, 10 mM NH_4OH containing 0.02% SDS.[1] To remove higher molecular mass proteins, such as albumin and immunoglobulin G, the samples were ultrafiltered using Centrisart ultracentrifugation devices (20 kDa MWCO; Sartorius, Göttingen, Germany) at 2,000 g relative centrifugal force until 1.1 mL of filtrate was obtained. This filtrate was then applied onto a PD-10 desalting column (GE Healthcare, Uppsala, Sweden) equilibrated in 0.01% NH_4OH in HPLC-grade H_2O (Roth, Germany) to decrease matrix effects by removing urea, electrolytes, and salts, and to enrich peptides. Finally, all samples were lyophilized, stored at 4 ºC, and suspended in HPLC-grade H_2O shortly before capillary electrophoresis coupled with mass spectrometry (CE-MS).

As described in detail elsewhere,[2] CE-MS analyses were performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Brea, CA) on-line coupled to a micrOTOF MS (Bruker Daltonic, Bremen, Germany). The ESI sprayer (Agilent Technologies, Palo Alto, CA) was grounded, and the ion spray interface potential was set between -4 and -4.5 kV. Data acquisition and mass spectrometry acquisition methods were automatically controlled by the capillary electrophoresis via contact-close-relays. Spectra were accumulated every 3 seconds, over a range of charge states (m/z) 350 to 3000. Previous publications described the accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE-MS measurements in detail. [2,3]

Mass spectra were processed using MosaiquesVisu software, including peak picking, deconvolution and deisotoping.[4] Migration time and peak intensity were normalized using internal peptide standards.[5] These fragments result from normal biological processes and

appear to be unaffected by any disease state studied to date based on over 20,000 samples in the Mosaiques database.[6] The resulting list of peaks characterizes each peptide by its molecular mass, normalized capillary electrophoresis migration time, and normalized signal intensity. All detected peptides were deposited, matched, and annotated in a Microsoft SQL database, allowing further analysis and comparison of multiple patient groups. Peptide fragments were combined into a single summary variable, using the support-vector machine based MosaCluster software, version 1.6.5.

In the present study, we used the previously published multidimensional classifier, which is associated with decreased left ventricular function and which combines information from 85 peptide fragments identified in 19 patients with diastolic LV dysfunction and 19 controls.[7] The classifier established by SVM modelling allows the classification of samples in the high dimensional data space. MosaCluster calculates classification scores based on the amplitudes of all HF1 biomarkers in the different samples. Classification is performed by determining the Euclidian distance (defined as the SVM classification score) of the vector to a maximal margin separating hyperplane. The SVM classifier uses the log transformed intensities of the urinary peptides as coordinates in an N-dimensional space. For HF1, N equals to 85. The SVM classifier then builds an N – 1 dimensional hyperplane that spans this space by performing a quadratic programming optimization of a Lagrangian, using the training labels only while allowing for samples to lie at the wrong side of the plane. For such mistakes in classification the SVM introduces a cost parameter C. Because non-separable problems in low dimensions may be separable in higher dimensions, the SVM uses the Kernel-trick to transform the data to a higher dimensional space. MosaCluster uses the standard radial basis functions as kernel. These functions are just Gaussians with the parameter gamma controlling their width. The optimal parameters C and gamma are found via cross validation error estimation, using a lattice build by different values of these two

parameters. SVMs are frequently implemented in data mining software. In particular, the kernlab cran R package is a versatile tool for building SVM based-classifiers.

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Table S1. List of 85 Peptides Included in the HF1 Biomarker

Peptide			Cases		Controls			
ID	Mass (Da)	CE Time (min)	%	MA	%	MA	R	*P*-value (Unadjusted)
81272	2211.98	33.23	0	0	0.42	2.67	0	1.99E-03
129821	3333.36	19.42	0	0	0.47	2.39	0	8.72E-04
8725	949.4	25.79	0.05	1.94	0.63	2.28	0.067	2.22E-04
123106	3130.43	30.82	0.05	1.98	0.47	2.63	0.080	2.57E-03
1577	840.41	23.17	0.05	1.65	0.47	1.85	0.095	3.29E-03
103493	2658.22	19.5	0.05	3.36	0.47	3.29	0.109	4.71E-03
44146	1518.6	19.37	0.11	1.91	0.58	2.49	0.145	1.33E-03
4845	900.27	43.66	0.16	1.55	0.63	2.44	0.161	1.33E-03
37610	1421.59	38.71	0.11	1.73	0.53	1.87	0.192	6.07E-03
83441	2248.97	33.69	0.11	3.45	0.53	3.56	0.201	4.88E-03
74703	2087.84	19.42	0.11	2.64	0.53	2.7	0.203	6.76E-03
101157	2616.16	28.39	0.11	1.97	0.53	1.98	0.206	6.76E-03
103022	2649.2	34.85	0.16	2.52	0.68	2.56	0.232	2.50E-03
57360	1734.66	19.9	0.16	2.2	0.58	2.24	0.271	1.03E-02
46091	1554.66	28.59	0.16	2.08	0.53	2.24	0.280	1.18E-02
32022	1319.58	20.89	0.21	1.99	0.58	2.21	0.326	1.57E-02
102269	2638.18	28.42	0.26	2.3	0.68	2.49	0.353	1.26E-02
82708	2235.04	34.17	0.32	2.57	0.84	2.68	0.365	2.53E-03
188895	11967.55	20.47	0.26	2.68	0.63	2.94	0.376	9.50E-03
98089	2559.18	19.41	0.32	2.97	0.84	3	0.377	3.76E-03
138143	3593.47	20.2	0.26	2.67	0.68	2.68	0.381	1.50E-02
167786	4771.07	20.2	0.37	2.74	0.79	3.13	0.410	4.34E-03
61984	1835.71	19.91	0.53	2.64	1	3.12	0.448	1.33E-04
46369	1560.7	29.79	0.32	2.78	0.68	2.84	0.461	2.27E-02
143947	3801.77	33.46	0.37	2.26	0.79	2.24	0.473	2.67E-02
39275	1445.62	37.36	0.47	2.59	0.79	2.96	0.521	4.87E-03
56493	1716.66	20.18	0.47	2.56	0.79	2.74	0.556	2.11E-02
41972	1478.61	39.3	0.53	2.75	0.84	2.95	0.588	3.16E-03
24168	1195.48	37.51	0.58	2.8	0.84	3.26	0.593	3.12E-03
107858	2751.34	29.23	0.63	2.36	0.89	2.69	0.621	3.00E-03
23356	1179.52	37.49	0.58	2.63	0.84	2.9	0.626	2.67E-02
97599	2547.99	21.44	0.58	2.59	0.89	2.66	0.635	3.15E-02
8695	949.22	34.33	0.53	2.46	0.68	3.01	0.637	2.78E-02

Peptide			Cases		Controls			
ID	Mass (Da)	CE Time (min)	%	MA	%	MA	R	*P*-value (Unadjusted)
23697	1186.53	22.39	0.68	2.8	1	2.88	0.661	2.08E-02
36566	1401.38	36.56	0.58	2.77	0.74	3.27	0.664	8.74E-03
153832	4196.75	20.84	0.68	2.41	0.95	2.59	0.666	4.93E-03
26670	1235.56	26.65	0.63	3.02	0.84	3.3	0.686	1.08E-02
58050	1749.81	30.61	0.63	2.57	0.84	2.79	0.691	3.04E-02
28005	1255.48	35.77	0.68	3.08	0.84	3.4	0.733	3.19E-02
159396	4409.89	20	0.74	2.72	0.84	3.23	0.742	2.68E-02
69979	1996.79	20.98	0.79	2.86	0.95	3.17	0.750	8.53E-03
40737	1462.62	39.42	0.84	3.33	1	3.68	0.760	2.62E-04
65368	1901.82	43.83	0.79	3.17	0.89	3.61	0.779	1.52E-02
128086	3286.55	30.92	0.79	3.13	0.89	3.51	0.792	6.91E-04
73434	2067.82	20.62	0.84	3.1	1	3.28	0.794	1.42E-02
148086	3986.65	20.6	0.84	3.53	0.95	3.82	0.817	2.75E-03
108574	2764.21	42.63	0.79	3.56	0.89	3.85	0.821	2.43E-02
90344	2377.1	20.8	0.89	3.12	0.95	3.46	0.845	1.95E-02
36759	1405.61	39.04	0.89	2.94	0.95	3.18	0.866	1.02E-02
147541	3968.6	21.09	0.89	3.14	0.89	3.57	0.880	1.77E-03
28561	1265.59	27.09	0.89	3.36	0.89	3.79	0.887	1.10E-02
107460	2742.25	28.98	0.95	2.91	1	3.11	0.889	1.19E-02
32171	1321.59	28.37	0.95	4.07	1	4.27	0.906	1.82E-02
39322	1446.64	39.43	1	3.2	1	3.49	0.917	3.19E-02
35339	1378.61	28.82	1	3.36	1	3.53	0.952	1.54E-02
81196	2210.95	33.61	1	3.72	1	3.59	1.036	2.15E-02
41601	1469.67	23.69	1	3.72	1	3.56	1.045	2.33E-02
62866	1854.81	40.92	1	3.89	1	3.71	1.048	1.98E-02
99021	2570.19	42.56	1	3.88	1	3.7	1.049	1.19E-02
79136	2175	33.28	1	3.74	1	3.49	1.072	1.09E-02
50840	1623.73	24.12	0.95	4.17	0.95	3.86	1.080	9.77E-03
72533	2046.92	32.58	0.95	3.49	0.95	3.21	1.087	1.06E-02
57537	1737.78	23.73	1	4.02	0.95	3.82	1.108	2.15E-02
50212	1613.82	23.99	0.89	2.7	0.89	2.43	1.111	3.30E-02
60149	1794.8	23.92	1	3.72	0.95	3.47	1.128	6.20E-03
103198	2654.19	23.92	0.89	2.94	0.89	2.47	1.190	5.52E-03
104786	2679.2	23.53	1	3.58	0.89	3.34	1.204	7.89E-03

Peptide			Cases		Controls			
ID	Mass (Da)	CE Time (min)	%	MA	%	MA	R	*P*-value (Unadjusted)
33135	1338.6	23.99	1	2.86	0.89	2.65	1.213	1.20E-02
73291	2064.92	24.46	0.84	2.75	0.79	2.37	1.234	3.25E-02
45021	1532.62	26.35	1	2.82	0.89	2.55	1.243	1.67E-02
99475	2577.25	24.67	0.95	2.78	0.89	2.38	1.247	6.05E-03
40294	1452.66	23.61	1	2.85	0.84	2.62	1.295	2.17E-03
35424	1380.64	23.83	0.95	2.79	0.79	2.56	1.311	7.17E-03
131294	3375.57	31.92	1	2.87	0.79	2.71	1.341	1.80E-02
111564	2841.26	24.54	0.89	3.21	0.79	2.67	1.354	4.98E-03
104195	2663.2	23.51	0.89	2.61	0.74	2.29	1.371	2.07E-02
28747	1268.57	27.25	1	3.44	0.74	3.32	1.400	1.01E-02
44802	1526.69	23.92	0.79	2.51	0.63	2.1	1.499	1.10E-02
113452	2889.35	24.08	0.89	2.47	0.58	2.29	1.655	7.34E-03
69681	1989.88	32.44	0.84	2.43	0.42	2.51	1.936	2.03E-02
55516	1696.72	23.95	0.79	2.54	0.42	2.39	1.999	1.59E-02
80360	2196.02	33.16	0.68	2.74	0.26	2.73	2.625	1.15E-02
82784	2236.98	27.14	0.63	2.28	0.21	2.31	2.961	1.29E-02
56806	1723.52	37.74	0.53	2.31	0.11	2.52	4.417	1.03E-02
129182	3320.51	24.25	0.47	2.07	0.05	2.1	9.266	4.71E-03

ID, peptide identifier (SQL number); %, percentage of samples, in which the peptide could be detected; MA, mean signal amplitude of the peptides. R was calculated as ∑ (ln signal amplitude x frequency/number of participants) in cases divided by ∑ (ln signal amplitude x frequency/number of participants) in controls. The peptides were ordered by ascending R. Reproduced from reference 7.

Table S2. List of Peptides Included in HF1 with Available Information on Amino-Acid Sequence

ID	Sequence of Peptide	Protein Name	Cases		Controls		
			%	MA	%	MA	R
8725	GDAGSKGpmV	Collagen alpha-1(V)	0.05	1.94	0.63	2.28	0.067
123106	RDVEEEEEEEGLEEDAELLTELQEVLG	Coiled-coil and C2 domain-containing protein 1B	0.05	1.98	0.47	2.63	0.080
1577	KGDTGPpGP	Collagen alpha-1(III)	0.05	1.65	0.47	1.85	0.095
103493	DEAGSEADHEGTHSTKRGHAKSRPV	Fibrinogen alpha	0.05	3.36	0.47	3.29	0.109
44146	DDFDAHKALEDDE	Isoform 1 of Histone-lysine N-methyltransferase MLL2	0.11	1.91	0.58	2.49	0.146
4845	GGSGAmGSmD	Immunoglobulin-like and fibronectin type III domain-containing protein 1	0.16	1.55	0.63	2.44	0.161
37610	GPpGpPGSpGEQGPSG	Collagen alpha-1(I)	0.11	1.73	0.53	1.87	0.192
83441	GAVGEKGEPGEAGEpGLpGEGGPpG	Collagen alpha-1(V)	0.11	3.45	0.53	3.56	0.201
74703	KSSSHQDSSRmSSVGDYNT	Bone morphogenetic protein 5	0.11	2.64	0.53	2.7	0.203
101157	GPpGADGQpGAKGEpGDAGAKGDAGpPGPA	Collagen alpha-1(I)	0.11	1.97	0.53	1.98	0.207
103022	FNINNLDNNWLKMHFWFYYA	Dermatan-sulfate epimerase-like protein	0.16	2.52	0.68	2.56	0.232
46091	KGETGDVGQMGppGPP	Collagen alpha-1(V)	0.16	2.08	0.53	2.24	0.280
32022	TYFPHFDLSHG	Hemoglobin subunit alpha	0.21	1.99	0.58	2.21	0.326
82708	GRTGDAGPVGPPGPpGppGpPGPPS	Collagen alpha-1(I)	0.32	2.57	0.84	2.68	0.365
98089	DEAGSEADHEGTHSTKRGHAKSRP	Fibrinogen alpha	0.32	2.97	0.84	3	0.377
61984	DQDKHDDSTDDSDTDK	WW domain-binding protein 11	0.53	2.64	1	3.12	0.448
46369	GPpGEKGGQGPPGpQGp	Collagen alpha-1(V)	0.32	2.78	0.68	2.84	0.461
143947	DQGPVGRTGEVGAVGPpGFAGEKGPSGEA GTAGPpGTpGPQG	Collagen alpha-2(I)	0.37	2.26	0.79	2.24	0.472
39275	DGVGQpGLPGpPGPpG	Collagen alpha-1(XVIII)	0.47	2.59	0.79	2.96	0.520
56493	KGDEGEAGDPGDDNNDI	Collagen alpha-1(VI)	0.47	2.56	0.79	2.74	0.556
41972	EQGLpGAAGQDGPpGP	Isoform D preproprotein of collagen alpha-1 (XI)	0.53	2.75	0.84	2.95	0.588
24168	GPpGPPGPSSNQG	Collagen alpha-6(IV)	0.58	2.8	0.84	3.26	0.593
107858	VSESSIHIIGVSLGAHVGGmVGQLFGGQ	Isoform 2 of phospholipase A1 member A	0.63	2.36	0.89	2.69	0.621

ID	Sequence of Peptide	Protein Name	Cases		Controls		
			%	MA	%	MA	R
23356	GPpGPpGPSSNQG	Collagen alpha-6(IV)	0.58	2.63	0.84	2.9	0.626
97599	LGSHSQDEEDEDTEYFDAMEDS	101 kDa protein	0.58	2.59	0.89	2.66	0.634
23697	DDGEAGKpGRpG	Collagen alpha-1(I)	0.68	2.8	1	2.88	0.661
36566	EEEDSSDSSSDSE	Isoform 1 of AP-3 complex subunit beta-1	0.58	2.77	0.74	3.27	0.664
26670	GQDGRpGPpGPpG	Collagen alpha-1(I)	0.63	3.02	0.84	3.3	0.686
58050	GPpGEAGKpGEQGVPGDLG	Collagen alpha-1(I)	0.63	2.57	0.84	2.79	0.691
28005	TYFPHFDLSHG	Hemoglobin subunit alpha	0.68	3.08	0.84	3.4	0.733
69979	KGSpGSDGpKGEKGDPGpEGP	Isoform 2C2A' of collagen alpha-2(VI) chain	0.79	2.86	0.95	3.17	0.750
40737	GPpGPAGNpGpSpNSP	Isoform 1 of collagen alpha-1(XXVI)	0.84	3.33	1	3.68	0.760
65368	WIDAPDDVFYMATEET	Metastasis-associated protein MTA1 79 kDa protein	0.79	3.17	0.89	3.61	0.779
73434	ADGSDLDAVSHGSmDSGHGTH	C-myc promoter-binding protein isoform 1	0.84	3.1	1	3.28	0.794
108574	DmGPpGPQGPpGKDGPPGVKGENGHPGSP	Isoform 2 of collagen alpha-1 (XIII)	0.79	3.56	0.89	3.85	0.821
90344	GKNGDDGEAGKpGRpGERGPpGPQ	Collagen alpha-1(I)	0.89	3.12	0.95	3.46	0.845
36759	PpGPpGFPGDpGPpG	Collagen alpha-3(V)	0.89	2.94	0.95	3.18	0.866
28561	SpGPDGKTGPpGPA	Collagen alpha-1(I)	0.89	3.36	0.89	3.79	0.886
107460	KNGETGPQGPPGPTGPGGDKGDTGPpGpQG	Collagen alpha-1(III)	0.95	2.91	1	3.11	0.889
32171	ApGDRGEpGPpGPA	Collagen alpha-1(I)	0.95	4.07	1	4.27	0.905
39322	GPpGPpGFPGDPGPpG	Collagen alpha-3(V)	1	3.2	1	3.49	0.917
35339	ApGEDGRpGPpGPQ	Collagen alpha-1(II)	1	3.36	1	3.53	0.952
81196	NGApGNDGAKGDAGApGApGSQGApG	Collagen alpha-1(I)	1	3.72	1	3.59	1.036
41601	DGQPGAKGEpGDAGAK	Collagen alpha-1(I)	1	3.72	1	3.56	1.045
62866	SGpQGppGSEGFTGPPGPQG	Collagen alpha-2(IV)	1	3.89	1	3.71	1.048
99021	QQEQLQQQQFQQQQEQLQQQ	Zinc finger protein 853	1	3.88	1	3.7	1.049
79136	AGPpGEAGKpGEQGVpGDLGApGP	Collagen alpha-1(I)	1	3.74	1	3.49	1.072
50840	DGApGKNGERGGpGGpGP	Collagen alpha-1(III)	0.95	4.17	0.95	3.86	1.080
72533	PpGEAGKpGEQGVpGDLGApGP	Collagen alpha-1(I)	0.95	3.49	0.95	3.21	1.087

ID	Sequence of Peptide	Protein Name	Cases		Controls		
			%	MA	%	MA	R
57537	NDGApGKNGERGGpGGpGP	Collagen alpha-1(III)	1	4.02	0.95	3.82	1.108
50212	VGGGEQPPPAPAPRRE	Xylosyltransferase 1	0.89	2.7	0.89	2.43	1.111
60149	GNDGApGKNGERGGpGGpGP	Collagen alpha-1(III)	1	3.72	0.95	3.47	1.128
103198	ERGEAGIpGVpGAKGEDGKDGSpGEpGA	Collagen alpha-1(III)	0.89	2.94	0.89	2.47	1.190
104786	NRGERGSEGSPGHpGQpGppGpPGAPGP	Collagen alpha-1(III)	1	3.58	0.89	3.34	1.204
33135	GAPGPRGRDGEpGT	Isoform 1 of collagen alpha-1(II)	1	2.86	0.89	2.65	1.213
73291	DDKDEEDSPRPRSPPGGPD	Zinc finger and BTB domain-containing protein 46	0.84	2.75	0.79	2.37	1.234
45021	RDGEPGTPGNpGPpGP	Isoform 1 of collagen alpha-1(II)	1	2.82	0.89	2.55	1.243
99475	DDILASPPRLPEPQPYPGAPHHSS	Collagen alpha-1(XVIII)	0.95	2.78	0.89	2.38	1.246
40294	DEPPQSPWDRVK	Apolipoprotein A-I	1	2.85	0.84	2.62	1.295
35424	AMFGPKGFGRGGAE	Cysteine-rich protein 1	0.95	2.79	0.79	2.56	1.311
131294	PGEDGEpGRNGNPGEVGFAGSpGARGFPGAPGLPGL	Collagen alpha-2(V)	1	2.87	0.79	2.71	1.341
111564	ERGEAGIpGVpGAkGEDGKDGSpGEpGANG	Collagen alpha-1(III)	0.89	3.21	0.79	2.67	1.354
104195	NRGERGSEGSPGHPGQPGPpGppGApGP	Collagen alpha-1(III)	0.89	2.61	0.74	2.29	1.371
28747	SpGERGETGPpGP	Collagen alpha-1(III)	1	3.44	0.74	3.32	1.400
44802	GGAGEpGKNGAKGEpGp	Isoform 1 of collagen alpha-1(III)	0.79	2.51	0.63	2.1	1.499
113452	NGEAGSAGPpGppGLRGSpGSRGLPGADGRAG	Collagen alpha-2(I)	0.89	2.47	0.58	2.29	1.655
69681	SNGNpGPpGPSGSpGKDGPpGP	Collagen alpha-1(III)	0.84	2.43	0.42	2.51	1.936
55516	RGSGGGGGGGGQGSTNYGKS	Isoform 3 of heterogeneous nuclear ribonucleoprotein A/B	0.79	2.54	0.42	2.39	1.999
80360	ISVPGPMGPSGPRGLpGPpGApGP	Collagen alpha-1(I)	0.68	2.74	0.26	2.73	2.625
82784	ADGQpGAkGEpGDAGAKGDAGPpGP	Collagen alpha-1(III)	0.63	2.28	0.21	2.31	2.961

ID, peptide identifier (SQL number); %, percentage of samples, in which the peptide could be detected; MA, mean signal amplitude of the peptides. R was calculated as ∑ (ln signal amplitude x frequency/number of participants) in controls divided by ∑ (ln signal amplitude x frequency/number of participants) in cases. The peptides were ordered by ascending R.
Reproduced from reference 7.

Table S3. Fatal and Nonfatal Cardiovascular Events

Endpoint	Type	Number of events
Stroke	Fatal	3
	Nonfatal	3
Transient ischaemic attack	Nonfatal	5
Myocardial infarction	Fatal	2
	Nonfatal	3
Acute coronary syndrome	Nonfatal	1
Angina pectoris	Nonfatal	1
Ischaemic cardiomyopathy	Nonfatal	17
Coronary revascularisation	Nonfatal	16
Congestive heart failure	Fatal	5
	Nonfatal	16
Atrial fibrillation/arrhythmia	Nonfatal	6
Atrioventricular block	Nonfatal	3
Aortic aneurysm	Fatal	1
	Nonfatal	2
Pulmonary heart disease	Nonfatal	1
Arterial embolism	Nonfatal	1
Peripheral arterial diseases	Nonfatal	9
Total number		95

Follow-up of the 791 participants spanned a median of 6.1 years (5th to 95th percentile interval, 3.7–7.4). A participant can experience multiple nonfatal events, so that nonfatal events do not add up. In the outcome analyses, only the first event within each category was considered.

Table S4. Crude Event Rates by Thirds of the HF1 Distribution

Endpoint (number of events)	<–1.42 (n=264)		–1.42 to –0.68 (n=263)		≥ –0.68 (n=264)	
	n	Rate (95% CI)	n	Rate (95% CI)	n	Rate (95% CI)
Total mortality (n=35)	7	4.7 (4.6, 4.8)	9	5.9 (5.7, 6.0)	19	12.5 (12.4, 12.7)
Cardiovascular events (n=63)	8	5.4 (5.3, 5.5)	17	11.4 (11.2, 11.6)	38	26.9 (26.6, 27.2)
Cardiac events (n=45)	4	2.7 (2.6, 2.8)	13	8.7 (8.5, 8.8)	28	19.4 (19.1, 19.6)
Coronary events (n=22)	3	2.0 (1.9, 2.1)	4	2.6 (2.5, 2.7)	15	10.2 (10.0, 10.4)

Rates (95% confidence interval) are expressed as number of events per 1000 person-years.

Table S5. Covariables Associated with Cardiovascular and Cardiac Risk Selected by a Step-Down Procedure

Selected covariables	Cardiovascular events		Cardiac events	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Being female (0,1)	0.57 (0.34–0.95)	0.031	0.56 (0.31–1.03)	0.063
Age (+15.7 years)	3.63 (2.55–5.17)	<0.0001	3.21 (2.04–5.07)	<0.0001
Fasting plasma sugar (+0.78 mmol/L)	1.17 (0.99–1.39)	0.063	1.30 (1.10–1.52)	0.002
Smoking (0, 1)	2.57 (1.43–4.60)	0.002	1.83 (0.89–3.78)	0.10
On treatment with vasodilators (0, 1)	2.04 (1.03–4.02)	0.040	2.86 (1.39–5.91)	0.005

For age and fasting plasma glucose, hazard ratios express the risk per 1-SD increase in the explanatory variable. Models accounted for clustering of failure times within pedigrees. The baseline characteristics considered as potential covariables were sex, age, body mass index, systolic blood pressure, fasting plasma glucose, the total-to-HDL cholesterol ratio, γ-glutamyltransferase (as index of alcohol intake), smoking, history of cardiovascular disease, and treatment with diuretics (thiazides, loop diuretics and aldosterone antagonists), β-blockers, inhibitors of the renin-angiotensin system (angiotensin-converting-enzyme inhibitors or angiotensin type-1 receptor blockers), vasodilators (calcium-channel blockers or α-blockers), or other antihypertensive drugs. Covariables to be retained in the analysis were identified by a step-down procedure, removing the least significant covariable at each step until all *P*-values of covariables were less than 0.15.

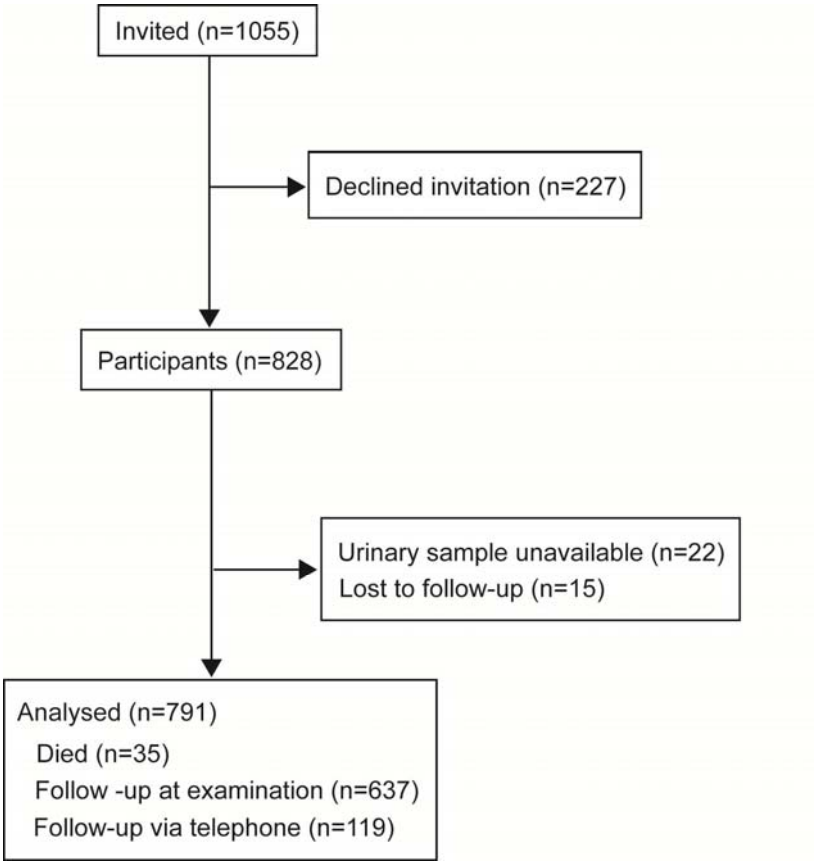

Figure S1. Flow chart.

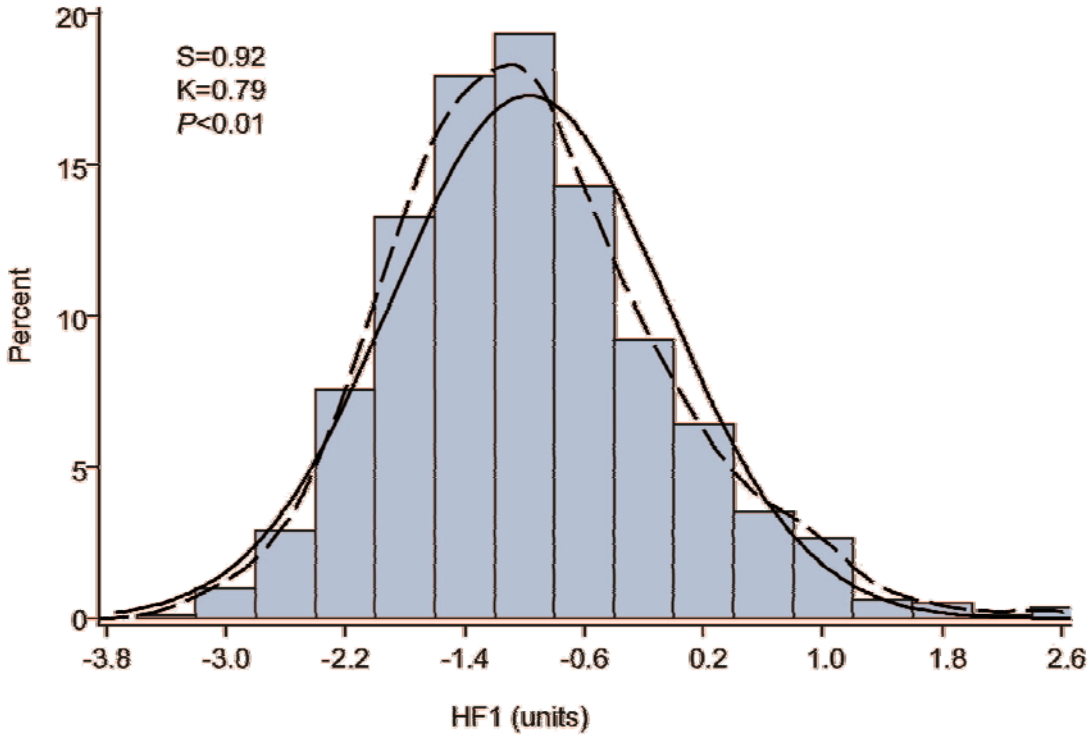

Figure S2. Distribution of the urinary multi-dimensional biomarker HF1 in 791 participants. The curves represent the fitted normal (full line) and kernel (dashed line) density plots. S and K are the coefficients of skewness and kurtosis, respectively. The P-value is for the Kolmogorov–Smirnov test and indicates departure from normality.

Urinary Proteome and Systolic Blood Pressure as Predictors of 5-Year Cardiovascular and Cardiac Outcomes in a General Population

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