Epidemiologic observations guiding clinical application of a urinary peptidomic marker of diastolic left ventricular dysfunction

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Abstract

Hypertension, obesity, and old age are major risk factors for left ventricular (LV) diastolic dysfunction (LVDD), but easily applicable screening tools for people at risk are lacking. We investigated whether HF1, a urinary biomarker consisting of 85 peptides, can predict over a 5-year time span mildly impaired diastolic LV function as assessed by echocardiography. In 645 white Flemish (50.5% women; 50.9 years [mean]), we measured HF1 by capillary electrophoresis coupled with mass spectrometry in 2005–2010. We measured early (E) and late (A) peak velocities of the transmitral blood flow and early (e’) and late (a’) mitral annular peak velocities and their ratios in 2009–2013. In multivariable-adjusted analyses, per 1-standard deviation increment in HF1, e’ was −0.193 cm/s lower (95% confidence interval: −0.352 to −0.033; \( P = .018 \)) and E/e’ 0.174 units higher (0.005–0.342; \( P = .043 \)). Of 645 participants, 179 (27.8%) had LVDD at follow-up, based on impaired relaxation.
in 69 patients (38.5%) or an elevated filling pressure in the presence of a normal (74 [43.8%]) or low (36 [20.1%]) age-specific E/A ratio. For a 1-standard deviation increment in HF1, the adjusted odds ratio was 1.37 (confidence interval, 1.07–1.76; \( P = .013 \)). The integrated discrimination (+1.14%) and net reclassification (+31.7%) improvement of the optimized HF1 threshold (−0.350) in discriminating normal from abnormal diastolic LV function at follow-up over and beyond other risk factors was significant (\( P \leq .024 \)). In conclusion, HF1 may allow screening for LVDD over a 5-year horizon in asymptomatic people. J Am Soc Hypertens 2018;12(6):438–447. © 2018 The Authors. Published by Elsevier Inc. on behalf of American Heart Association. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Keywords: Diastolic left ventricular function; population science; screening; urinary proteomics.

Introduction

Diastolic heart failure (DHF) represents half of all heart failure cases\(^1\) and has a 30% death rate within 1 year of the first hospital admission.\(^2\) Subclinical left ventricular (LV) diastolic dysfunction (LVDD) has 25% prevalence in the general population.\(^3,4\) Hypertension, obesity, old age, and insulin resistance are among the major risk factors.\(^3,4\) LVDD is an insidious condition evolving to DHF.\(^5\) Screening for LVDD at the point of entry in health care is extremely challenging because it requires awareness of predisposing risk factors, clinical interpretation of vague symptoms and signs, and LV imaging demonstrating functional or structural LV changes. The observation that natriuretic peptide levels in LVDD patients are often within normal limits\(^3,4,6\) complicates the matters further and justifies the quest for novel biomarkers specific for LVDD at an early stage long before it progresses to DHF.

Capillary electrophoresis coupled with high-resolution mass spectrometry (CE-MS) enables detection of over 5000 distinct peptides in urine samples.\(^7,8\) We previously identified a multidimensional urinary classifier, HF1, mainly consisting of dysregulated collagen fragments,\(^9,10\) which in case-control studies\(^11\) and in the general population\(^10,11\) was reproducibly associated with subclinical LVDD. In patients progressing from LVDD to DHF, the LV wall undergoes fibrosis characterized by increased interstitial deposition\(^12\) and cross-linking of collagen I at the detriment of collagen III.\(^13,14\) We hypothesized that urinary markers of collagen turnover, and circulating serum markers of collagen degradation might predict LVDD\(^3,4\) over and beyond known risk factors and might therefore represent easily applicable screening tools in primary care. We tested our hypothesis in participants enrolled in the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO\(^9,11\)), in whom we related the echocardiographically assessed diastolic LV function (2009–2013) to HF1, and the serum markers of collagen degradation measured approximately 5 years earlier (2005–2010).

Methods

Study Participants

FLEMENGHO is a family-based population study,\(^15,16\) which complies with the Helsinki declaration\(^17\) and received ethical approval from the Ethics Committee of the University Hospitals Leuven (approval number B32220083510). For the current analysis, we selected 655 people, whose urinary proteome had been measured in 2005–2010 (baseline) and who had undergone echocardiography in 2009–2013 (follow-up). The participation rate at echocardiography was 80.0%. We excluded 10 participants, whose diastolic LV function at follow-up could not be reliably assessed, because of atrial fibrillation (\( N = 6 \)) or paced heart rhythm (\( N = 4 \)). Thus, the number of participants statistically analyzed totaled 645.

Clinical Measurements

Body mass index (BMI) was weight in kilogram divided by height in meters squared. Waist circumference was determined using a measuring tape. Abdominal obesity was a waist circumference of \( \geq 88 \) cm in women and \( \geq 102 \) cm in men.\(^18\) Blood pressure was the average of five consecutive auscultatory readings. Hypertension was a blood pressure of \( \geq 140 \) mm Hg systolic or \( \geq 90 \) mm Hg diastolic or use of antihypertensive drugs. Estimated glomerular filtration rate (eGFR) was derived from serum creatinine (Cr) by the chronic kidney disease epidemiology collaboration equation.\(^19\) Diabetes mellitus was a self-reported diagnosis, a fasting plasma glucose (Glyc) \( \geq 7 \) mmol/L, or use of antidiabetic agents.\(^20\) Using validate questionnaires\(^21\) and published tables,\(^22\) we computed the energy spent in physical activity from body weight, time devoted to work, walking, sports and leisure time activities, and type of physical activity.

Echocardiography

Detailed information on the acquisition and offline analysis of the echocardiographic images is available in previous publications.\(^9,10\) In short, echocardiographic measurements, obtained with a Vivid7 Pro device (GE Vingmed, Horten, Norway) interfaced with a 2.5–3.5-MHz phased-array probe, were averaged over three heart cycles. Diastolic LV function was assessed by the EchoPac software, version 4.0.4 (GE Vingmed, Horten, Norway). In keeping with guidelines,\(^23\) we determined peak early (E) and late (A) diastolic velocities of the transmitral blood
flow from the pulsed Doppler signal and peak early (e') and late (a') mitral annular movement by tissue Doppler imaging with velocities averaged over four acquisition sites (septal, lateral, inferior, and posterior). Reproducibility across the four tissue Doppler imaging sampling sides ranged from 4.5% to 5.3% for e' and from 4.0% to 4.5% for a'. Patients with LVDD had an abnormally low age-specific transmitral E/A ratio indicative of impaired relaxa-

Biomarkers

Participants collected 24-hour urine samples within 1 week of the echocardiographic examination at the field center. For measurement of HF1 and microalbuminuria, aliquots (0.7 mL) were stored at −80°C for 8 years (median) and thawed immediately before analysis. Detailed information about urine sample preparation, proteome analysis by CE-MS, data processing, and sequencing has been published before.7,8 Peptides were combined into a single summary variable, using the MosaCluster software, version 1.6.5. HF1 was originally derived in a case-control study including participants with mild and moderate LVDD. It consists of 85 peptides (Table S1), mainly collagen fragments. HF1 is a robust9,10 urinary biomarker validated before in case-control studies9 and in the general popula-
tion.10 HF1 is normally distributed, higher values being associated with worse outcomes.10,24,25

At baseline (2005–2010), venous blood samples were drawn after at least 8 hours of fasting. Carboxyterminal telopeptide of collagen I (CITP) and tissue inhibitor of the matrix metalloproteinase type I (TIMP-I) were measured as circulating markers of collagen I degradation in 607 participants (94.1%). CITP was quantified by a quantitative enzyme immunoassay (Orion Diagnostica, Espoo, Finland) and TIMP-I (GE Healthcare Life Sciences, Buckinghamshire, UK) by sandwich enzyme-linked immunosorbent assay.14 The detection limits were 0.3 μg/L for CITP (inter-

Statistical Analysis

For database management and statistical analysis, we used the SAS system, version 9.4 (SAS Institute Inc., Cary, NC). Means were compared using the large-sample z-test and proportions by Fisher’s exact test. We normalized the distributions of the energy spent in physical activity and 24-hour microalbuminuria by a logarithmic transformation. Our statistical methods also included multivariable-adjusted linear and logistic regression with as dependent variables the echocardiographic indexes reflecting diastolic LV function on a continuous or binary scale. We adjusted for baseline covariates of physiological relevance, identified in previous analyses, including sex, age, BMI, systolic and diastolic blood pressure, heart rate, serum Cr, fasting blood Glyc, and treatment with inhibitors of the renin sys-
tem and ß-blockers. In sensitivity analyses, we replaced BMI by waist circumference and additionally accounted for 24-hour microalbuminuria, energy spent in physical activity.21,22 We determined optimal discrimination limits for HF1 by maximizing Youden’s index (the maximum of sensitivity plus specificity minus 1). Finally, we assessed the incremental value of the urinary biomarkers in discriminating between normal and abnormal LV function, using the integrated discrimination improvement (IDI) and the net reclassification improvement (NRI).26

Results

Baseline Characteristics of Participants

Of 645 participants, 326 (50.5%) were women. Mean values (standard deviation) were 50.9 ± 14.7 years for age, 26.5 ± 4.4 kg/m² for BMI, 103.5 ± 8.9 cm for waist circumference, and 128.8 ± 16.8/79.8 ± 9.3 mm Hg for systolic/diastolic blood pressure. Of all participants, 268 (41.6%) had hypertension, of whom 160 (59.7%) were on antihypertensive drug treatment; 18 (2.8%) had a history of diabetes mellitus; and 30 (4.7%) reported previous coronary heart disease. The antihypertensive drug classes used at baseline were diuretics in 59 (9.2%), ß-blockers in 100 (15.5%), inhibitors of the renin-angiotensin system in 51 (7.9%), and calcium-channel blockers in 26 (4.0%) participants. Table 1 lists the characteristics of participants by quartiles of the HF1 distribution. Age, BMI, waist circumference, systolic pressure, the prevalence of hypertension, the use of antihypertensive drugs, or being overweight, plasma Glyc, serum Cr, and TIMP-I all increased (P ≤ .004) with higher HF1 category, whereas the opposite was the case for eGFR (P < .0001). Across the HF1 quartiles, there were no differences (P ≥ .12) in the prevalence of abdominal obesity, the energy spent in physical activity, or 24-hour microalbuminuria.

Continuous Analyses

Median follow-up time was 4.8 years (interquartile range, 4.4–5.1 years; 5th–95th percentile interval, 3.7–5.4 years). Table S2 shows that the left atrial volume index, A', and a' peak velocities, and the E/e' ratio were greater (P ≤ .010) with higher baseline HF1 category, whereas
Biochemical data baseline age having the biggest impact (Figure 1, panels tions of e’ and E/e’ at follow-up with baseline HF1, riables (Figure 1, panels C and D) weakened the associa-
C and D). With all adjustments applied, per 1-standard systematic analysis, we replaced BMI by waist circumference
the opposite was the case (P < .0001) for the E and e’ peak velocities and the E/A and e’/a’ ratios. e’ (r = −0.79; Figure 1, panel A), E/e’ (r = 0.54; Figure 1, panel B), and HF1 (r = 0.36) were strongly dependent on age (P < .0001). Stepwise cumulative adjustment for the cova-
limits, score Categories of the Urinary HF1 Biomarker P
Limits, Score
< −1.623 −1.623 to −0.1047 −1.046 to −0.445 > −0.445
Number of subjects (%) All patients in category 162 161 161 161
Women 83 (51.2) 84 (52.2) 85 (52.8) 74 (46.0) .60
Smokers 40 (24.7) 33 (20.5) 22 (13.7) 26 (16.2) .057
Drinking alcohol 60 (37.0) 57 (35.4) 61 (37.9) 60 (37.3) .60
Hypertension 44 (27.2) 60 (37.3) 62 (38.5) 102 (63.4) < .0001
Antihypertensive treatment 18 (11.1) 27 (16.8) 38 (23.6) 77 (47.8) < .0001
Body mass index ≥25 kg/m² 81 (50.0) 95 (59.0) 99 (61.5) 125 (77.6) < .0001
Abdominal obesity 122 (75.3) 131 (81.4) 126 (78.3) 133 (82.6) .37
History of coronary heart disease 3 (1.9) 10 (6.2) 7 (4.4) 14 (8.7) .043
Diabetes mellitus 2 (1.2) 0 4 (2.5) 12 (7.5) .0003
Mean of characteristic Age, y 44.0 ± 14.0 49.9 ± 14.2 P < .001 52.0 ± 13.5 57.5 ± 13.9 P < .0001
Body mass index, kg/m² 25.3 ± 3.7 26.2 ± 3.7 P = 0.445 26.4 ± 4.5 28.3 ± 5.0 P < .0001
Waist circumference, cm 101.7 ± 7.8 102.9 ± 7.4 103.1 ± 9.9 106.2 ± 10.3 < .0001
Energy spent in physical activity, Kcal 1725 (1300–2108) 1788 (1400–2221) 1781 (1367–2207) 1809 (1411–2200) .60
Blood pressure Systolic pressure, mm Hg 125.2 ± 14.0 128.4 ± 18.9 128.1 ± 16.1 133.3 ± 17.2 P < .0003
Diastolic pressure, mm Hg 78.6 ± 8.8 79.6 ± 9.9 80.1 ± 9.2 81.0 ± 9.4 .13
Heart rate, beats per minute 60.2 ± 9.1 60.3 ± 9.6 60.0 ± 9.8 59.8 ± 10.6 .97
Biochemical data Total cholesterol, mmol/L 5.22 ± 1.51 5.45 ± 1.10 5.29 ± 0.95 5.29 ± 0.97 .36
Plasma glucose, mmol/L 4.83 ± 0.52 4.83 ± 0.43 4.92 ± 0.66 5.10 ± 1.23 .004
Serum creatinine, μmol/L 81.8 ± 13.4 83.4 ± 12.9 83.5 ± 13.0 88.1 ± 21.1* .002
eGFR, ml/min/1.73 m² 87.5 ± 14.9 82.1 ± 16.5* 80.6 ± 13.8 75.8 ± 16.3 < .0001
24-h microalbuminuria, mg 5.29 (3.89–6.59) 5.73 (3.79–7.10) 5.83 (4.39–7.35) 6.38 (4.30–7.91) .12
CITP, μg/L 5.44 ± 1.62 5.30 ± 1.80 5.63 ± 2.00 5.82 ± 2.56 .13
TIMP-I, ng/mL 603 ± 154 670 ± 192* 679 ± 154 730 ± 224* < .0001
NT-proBNP, pmol/L 212 (158–284)* 199 (143–288) 217 (148–318) 202 (130–284) .51

The corresponding association sizes were −0.005 (CI, −0.159 to 0.148; P = .94) for CITP and −0.042 (CI, −0.205 to 0.120; P = .61) for TIMP-I. When in the sensi-
tivity analysis, we replaced BMI by waist circumference and additionally adjusted for energy spent in physical
activity analysis, we replaced BMI by waist circumference
Abdominal obesity was a waist circumference of ≥88 cm in women and ≥102 cm in men. Blood pressure was the average of five consecutive auscultatory readings. Heart rate was determined after
15 minutes recumbent rest. Hypertension was a blood pressure of ≥140 mm Hg systolic or ≥90 mm Hg diastolic, or use of antihypertensive drugs. CITP and TIMP-I were available in 149, 153, 155, and 150 participants of the 1st, 2nd, 3rd, and 4th quartile, respectively (607 in total). NT-proBNP was measured in 144, 149, 150, and 148 participants of the 1st, 2nd, 3rd, and 4th quartile, respectively (591 in total). Means are arithmetic means (SD) or geometric means (interquartile range). P values denote the significance of the differences in prevalence rates or means across quartiles of the HF1 distribution.

* Significance of the difference with the adjacent lower quartile: P ≤ .05.
† P ≤ .01.
‡ P ≤ .001.
§ P < .0001.
activity and 24-microalbuminuria, $e'$ was 0.215 cm/s lower (CI, 0.377 to 0.053; $P = .009$) and $E/e'$ 0.179 units higher (CI, 0.009–0.349; $P = .039$). None of the other echocardiographic variables (E, A, E/A, $e'/a'$) was associated with HF1, CITP, or TIMP-I.

**Categorical Analyses**

Next, we analyzed the relative risk of LVDD in relation to HF1. Of 645 participants, 179 (27.8%) had LVDD at follow-up, based on impaired relaxation in 69 patients (38.5%) or an elevated filling pressure in the presence of a normal (74 [43.8%]) or low (36 [20.1%]) age-specific E/A ratio. The probability of having LVDD curvilinearly increased with age (model $R^2 = 0.32$; Figure 2, panel A). Age strongly influenced the parameter estimates for the association of the risk of LVDD with baseline HF1 (Figure 2, panel B). In multivariable-adjusted analyses, per 1-standard deviation increment, the odds ratios were 1.37 (CI, 1.07–1.76; $P = .013$) for HF1, 1.16 (CI, 0.91–1.49; $P = .13$) for CITP, and 1.26 (CI, 0.96–1.66; $P = .09$) for TIMP-I. For a doubling of NT-proBNP, the odds ratio was 1.16 (CI, 0.87–1.55; $P = .30$). HF1 in the presence of NT-proBNP yielded an odds ratio of 1.39 (CI, 1.07–1.81; $P = .013$). When in the sensitivity analysis, we replaced BMI by waist circumference and additionally adjusted for energy spent in physical activity and 24-microalbuminuria, the odds ratios were 1.41 (CI, 1.10–1.82; $P = .0068$) for HF1 and 1.43 (CI, 1.10–1.86; $P = .0071$) for HF1 in the presence of NT-proBNP.

**Added Diagnostic Accuracy**

From baseline to follow-up, HF1 increased from $-1.00 \pm 0.90$ to $-0.86 \pm 0.90$ ($P = .0004$). At baseline, the optimized HF1 threshold was $-0.350$. Among all participants, sensitivity was 43.0%, and specificity was 86.1%. Of 469 participants with baseline HF1 $< -0.350$, 90 (19.2%) progressed to $\geq -0.350$ at follow-up. Conversely, of 129 participants with baseline HF1 at baseline $\geq -0.350$, 62 (48.1%) reverted to a value of $< -0.350$ at follow-up. When stratified by age ($\geq 50$ versus $< 50$ years), BMI ($\geq 25$ versus $< 25$ kg/m$^2$) or hypertension versus normotension, sensitivity, and specificity

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**Figure 1.** Simple correlations of $e'$ (A) and $E/e'$ (B) with age. In panels A and B, red and blue dots indicate women and men, respectively. Full lines represent the regression slopes, and dotted lines represent the 95% confidence boundaries for the prediction of the mean values (blue) and individual values (red) of $e'$ and $E/e'$ at any given age. Associations of $e'$ (C) and $E/e'$ (D) with HF1 weaken as covariables were stepwise introduced in the regression model but remained significant after full adjustment. The association sizes are expressed for a 1-standard deviation increment in HF1. The shaded area denotes the 95% confidence boundary of the parameter estimates.

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**Figure 2.** Simple correlations of $e'$ (A) and $E/e'$ (B) with age. In panels A and B, red and blue dots indicate women and men, respectively. Full lines represent the regression slopes, and dotted lines represent the 95% confidence boundaries for the prediction of the mean values (blue) and individual values (red) of $e'$ and $E/e'$ at any given age. Associations of $e'$ (C) and $E/e'$ (D) with HF1 weaken as covariables were stepwise introduced in the regression model but remained significant after full adjustment. The association sizes are expressed for a 1-standard deviation increment in HF1. The shaded area denotes the 95% confidence boundary of the parameter estimates.
Figure 2. The probability of having LVDD curvilinearly increased with age (A). \( r_{\text{age}} \) and \( r_{\text{age}^2} \) are the partial correlation coefficients for the linear and squared terms of age and \( P_{\text{age}} \) and \( P_{\text{age}^2} \) the corresponding significance levels. \( R^2 \) is the coefficient of multiple determination. In panel A, red and blue dots indicate women and men, respectively. The full line represents the regression slope, and the dotted lines represent the 95% confidence boundaries for the prediction of the mean probabilities (blue) and individual probabilities (red) of LVDD at any given age. The odds of having LVDD in relation to HF1 weakened as covariables were stepwise introduced in the logistic model but remained significant after full adjustment (B). Odds ratios are expressed for a 1-standard deviation increment in HF1. The shaded area denotes the 95% confidence boundary of the parameter estimates. Stepwise cumulative were implemented for sex, age, BMI, SBP, DBP, heart rate (HR), serum creatinine (Crt), fasting blood glucose (Glyc), and treatment with inhibitors of the renin system (RAS) and \( \beta \)-blockers (BB).

Table 2
Classification parameters by HF1 at baseline

<table>
<thead>
<tr>
<th>Groups</th>
<th>Classification Parameters Using −0.350 as Optimized HF1 Threshold</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity (%)</td>
<td>Specificity (%)</td>
<td>Positive Predictive Value (%)</td>
<td>Negative Predictive Value (%)</td>
<td>Misclassification Rate</td>
<td>Integrated Discrimination Improvement (%)</td>
</tr>
<tr>
<td>All participants (N = 645)</td>
<td>43.0</td>
<td>86.1</td>
<td>54.2</td>
<td>79.7</td>
<td>25.9</td>
<td>1.14 (0.15 to 2.13)</td>
</tr>
<tr>
<td>Age ≥50 y (N = 345)</td>
<td>45.9</td>
<td>81.4</td>
<td>67.3</td>
<td>64.3</td>
<td>34.8</td>
<td>1.66 (0.30 to 3.02)</td>
</tr>
<tr>
<td>Age &lt;50 y (N = 300)</td>
<td>77.3</td>
<td>89.2</td>
<td>85.7</td>
<td>93.6</td>
<td>15.7</td>
<td>0.07 (−0.40 to 0.54)</td>
</tr>
<tr>
<td>BMI ≥25 kg/m² (N = 400)</td>
<td>43.1</td>
<td>82.3</td>
<td>59.6</td>
<td>70.3</td>
<td>32.6</td>
<td>0.81 (0.09 to 1.71)</td>
</tr>
<tr>
<td>BMI &lt;25 kg/m² (N = 245)</td>
<td>57.1</td>
<td>90.4</td>
<td>36.4</td>
<td>92.5</td>
<td>15.0</td>
<td>3.75 (−0.39 to 7.88)</td>
</tr>
<tr>
<td>Hypertension (N = 268)</td>
<td>47.3</td>
<td>76.3</td>
<td>64.9</td>
<td>60.9</td>
<td>37.7</td>
<td>0.78 (−0.29 to 1.85)</td>
</tr>
<tr>
<td>Normotension (N = 377)</td>
<td>68.0</td>
<td>90.2</td>
<td>66.7</td>
<td>89.7</td>
<td>17.5</td>
<td>2.77 (−0.18 to 5.73)</td>
</tr>
</tbody>
</table>

BMI, body mass index.

The basic reference models included as covariables sex, age, BMI, systolic and diastolic blood pressure, heart rate, serum creatinine, fasting blood glucose, and treatment with inhibitors of the renin system and \( \beta \)-blockers. The integrated discrimination improvement (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. Cases and controls are participants with and without LVDD, respectively. The net reclassification improvement (NRI) is the sum of the percentages of participants reclassified correctly as cases and controls.

* Significance: \( P \leq 0.05 \).
† \( P \leq 0.01 \).
‡ \( P \leq 0.001 \).
§ \( P \leq 0.0001 \).
were consistently higher in the low compared with the high-risk group, whereas the misclassification rate showed the opposite trend (Table 2). In all and aged participants, both IDI and NRI reached significance ($P \leq .024$), whereas this was also the case for NRI in overweight, normal weight, and normotensive people ($P \leq .007$).

**Discussion**

The objective of our present study was to evaluate whether the multidimensional urinary biomarker HF1 could discriminate over a 5-year horizon between normal LV function and mildly impaired LVDD. The key findings can be summarized as follows: (1) in continuous analyses, lower $e'$ and greater $E/e'$ at follow-up were associated with higher baseline HF1; (2) in categorical analyses, HF1 predicted subclinical LVDD and; (3) over a 5-years horizon, HF1 improved discrimination between people with normal and mildly impaired diastolic LV function. Two previous FLEMENGO reports\textsuperscript{25,27} illustrate the clinical relevance of the early diagnosis of LVDD. Higher HF1\textsuperscript{25} and lower $e'$\textsuperscript{27} over a median follow-up of 5–6 years predicted the incidence of cardiovascular complications. An unexpected finding was that over this short follow-up period, HF1 was more predictive than systolic blood pressure.\textsuperscript{25} This observation probably reflects the time course of events, systolic blood pressure being a major driver of LVDD. In the Framingham Heart Study, remote blood pressure (average of all reading 11–20 years before current) and recent blood pressure (average of all readings 1–10 years before current) predicted cardiovascular disease incrementally over current blood pressure.\textsuperscript{28} Explanations offered by the Framingham investigators were that antecedent blood pressure is a fore-runner of cardiovascular target organ damage, which is on the path to hard cardiovascular complications and that the relation between cardiovascular risk and blood pressure weakens over time, for instance by the initiation of antihypertensive drug treatment.\textsuperscript{28}

While the diagnosis of DHF remains challenging in a hospital environment, this is even more the case for asymptomatic LVDD at the point of entry in health care. Echocardiography is the diagnostic approach recommended by guidelines, which requires highly skilled observers and is costly and impossible to implement on a large scale. Hence, screening by means of biomarkers in primary care is an option to be favored. Figure 3 proposes how HF1 might be applied in clinical practice in asymptomatic high-risk individuals. In the presence of one or more clinical risk factors for LVDD, in particular the combination of seniority, overweight or abdominal obesity, and hypertension ($N = 162$; 25.1% of our study population), HF1 might be used as a screening tool. If the value is $< -0.350$, managing risk factors over a 5-year time span is the intervention to be recommended. In contrast, if HF1 is $\geq -0.350$, a second test might inform the health-care provider whether continuing managing risk factors for 5 years is sufficient or whether the patient should be referred for echocardiography. An added benefit is that HF1 predicts worsening of renal function\textsuperscript{25} and the 5-year incidence of cardiovascular and cardiac events.\textsuperscript{25} NT-proBNP is the biomarker most frequently used in clinical practice, but its distribution shows large overlap between individuals with normal diastolic LV function, LVDD, or even DHF.\textsuperscript{10,27} In our present study, in line with previous publications,\textsuperscript{3,4,6} NT-proBNP did not add to the prediction of LVDD over and beyond classical risk factors. Moreover, HF1 in the presence of NT-proBNP fully retained its prognostic value. Another issue to be considered is the risk assessment of individuals not at high risk. In such participants, HF1 values $\geq -0.350$ tended to have higher sensitivity than in high-risk individuals (Table 2). The large amount of prognostic information carried by old age and higher BMI (Figures 1 and 2) explain this observation. Thus, although a positive test in low-risk individuals is predictive, it cannot be recommended, because its application on a large scale is impracticable.

A major advantage of running proteomics on urine samples is the comfort for the patient because all what is needed is a fresh mid-morning urine sample of 5 mL. Urinary proteins remain stable for a time long enough to perform the proteome analysis in a reliable manner.\textsuperscript{29} Two independent sets of experiments demonstrated that the urinary proteome does not undergo significant changes when urine is stored for 3 days at 4°C\textsuperscript{30} or for 6 hours at room temperature.\textsuperscript{31} Moreover, for studies running over several years, urine can be stored at $-20^\circ$C without significant alteration of the proteome.\textsuperscript{29} The urinary proteome is well characterized, and reference standards are available.\textsuperscript{8} CE-MS that provides sufficient sensitivity and high reproducibility is capable to resolve up to 6000 different peptides per sample within approximately 45 minutes.\textsuperscript{32} On the other hand, urinary proteomic analysis remains substantially more costly than other diagnostic tests employed in the management of patients at risk of or already having LVDD. However, a cost-effectiveness analysis within the setting of the German health insurance system suggested that CKD273, a multidimensional classifier used for the early diagnosis of decline in the eGFR performs better than microalbuminuria.\textsuperscript{33} Markov models were constructed for diabetic patients free of chronic kidney disease or other diabetic complication and assumed follow-up from 45 until 85 years or death. By using CKD273 instead of microalbuminuria, the overall cost per patient was €17,567 ($20,731) lower, and the number of patients progressing to dialysis decreased by 30%.\textsuperscript{33} Whether or not the Markov models would materialize in clinical care is currently being tested in a randomized clinical trial.\textsuperscript{34} Our current and previous findings are in line with the pathophysiologic concepts underlying deterioration of diastolic LV function. In patients with LVDD, the LV wall
undergoes fibrosis characterized by increased interstitial deposition\textsuperscript{12} and cross-linking of collagen I at the detriment of collagen III.\textsuperscript{13,14} Small increases in the collagen I/III ratio augment myocardial stiffness, thereby reducing early diastolic LV filling and increasing LV filling pressure.\textsuperscript{35,36} Of the peptides with known amino acid sequence, which make up HF1, 60.0\%\textsuperscript{9} consist of dysregulated collagen fragments. Recent cross-sectional analyses of sequenced urinary peptides in FLEMENGO participants demonstrated that LVDD was associated with higher levels of urinary collagen I fragments, lower levels of urinary collagen III degradation products, and higher levels of circulating tissue inhibitor of matrix metalloproteinase type I, an inhibitor of collagen-degrading enzymes.\textsuperscript{11} Combined, these data suggest that HF1 reflects collagen degradation, and in this way reveals relevant molecular processes that subsequently lead to remodeling of the extracellular matrix and fibrosis of the myocardium.

Strong points of our study are the assessment of Doppler indexes as early signs of subclinical LVDD and the adjustment of our analyses for a large number of covariables measured simultaneously with the urinary biomarker. What our study additionally highlighted is the strong age dependency of both diastolic LV function and the HF1 marker and providing a justification for repeating the measurement of HF1 if its value is $\geq -0.350$, as is currently recommended for albuminuria in the field of chronic kidney disease.\textsuperscript{37} Of note, HF1 retained its prognostic significance over and beyond established LVDD risk factors, in particular age and abdominal obesity. In all and older participants, HF1 improved both IDI and NRI. However, our study must also be interpreted within the context of its limitations. Although as outlined cardiovascular outcome data\textsuperscript{25,27} add a perspective to our current observations, an observational study cannot fully prove the utility of a biomarker. Further validation of HF1 as screening tool in a randomized clinical trial is therefore necessary. Such approach is presently being implemented to validate CKD273\textsuperscript{38} in the multicenter double-blind placebo-controlled PRIORITY trial (proteomic prediction and renin-angiotensin aldosterone system inhibition prevention of early diabetic nephropathy in type II diabetic patients with normal albumin excretion).\textsuperscript{34} Second, notwithstanding the consistency of the association between LVDD and HF1 in a discovery and test case-control study\textsuperscript{9} and subsequently in the general population\textsuperscript{10} and the pathophysiological plausibility in mechanistic studies,\textsuperscript{11} replication in an independent cohort would enhance the clinical relevance of our findings. Third, a sensitivity ranging from 43.0\% to 77.3\% might be considered as low. However, ECG is a commonly used instrument with a sensitivity of only 35\% to diagnose echocardiographically confirmed LV hypertrophy.\textsuperscript{39} Finally, our present study cannot prove the cardiac origin of the urinary collagen fragments that contribute to HF1.
However, in a tissue proteomic study, we applied liquid chromatography-tandem mass spectrometry to analyze biopsies from explanted human hearts, 15 with ischemic cardiomyopathy, 14 with dilated cardiomyopathy, and 12 healthy donor hearts discarded from implantation (control). In both ischemic and dilated cardiomyopathy, the tissue proteomic signature consistently showed higher abundance of proteins involved in the organization of the extracellular matrix, which is in agreement with the contribution of dysregulated collagen fragments to HF1.

In conclusion, in a general population, HF1 allows screening for LVDD. Our current observations support the concept of porting easily obtainable multidimensional urinary biomarkers to clinical practice to enable a personalized approach to the diagnosis, prevention, and treatment of LVDD, a high-risk condition that affects 25% of the general population. Such biomarkers might be particularly useful in primary health care, particularly in older high-risk patients with hypertension or abdominal obesity and serve as a decision tool informing doctors when referral for echocardiography is indicated.

References


Expanded Methods

Participants collected 24-hour urine samples within 1 week of the clinical examination at the field center. Aliquots (0.7 mL) were stored at −80°C and thawed immediately before analysis. 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 2000 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% NH4OH in HPLC-grade H2O (Roth, Germany) to decrease matrix effects by removing urea, electrolytes, and salts and to enrich peptides. Finally, all samples were lyophilized, stored at 4°C, and suspended in HPLC-grade H2O shortly before capillary electrophoresis coupled with mass spectrometry (CE-MS).

As described in detail elsewhere,1–3 CE-MS analyses were performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Brea, California, USA) online coupled to a micrOTOF MS (Bruker Daltonics, Bremen, Germany). The ESI sprayer (Agilent Technologies, Palo Alto, CA, USA) 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) 350–3000. Previous publications described the accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE-MS measurements in detail.1–4 Mass spectra were processed using MosaiquesVisu software, including peak picking, deconvolution, and de-isotoping.5 Migration time and peak intensity were normalized, using internal polypeptide standards.6 These fragments result from normal biological processes and appear to be unaffected by any disease state. A strict correlation between peptide charge at the working pH of two and capillary electrophoresis migration time was used to avoid falsely characterized peptides.8

Peptide fragments identified in previous study were combined into a single summary variable, using the support-vector machine based MosaCluster software, version 1.6.5. As published previously,9 HF1 combined information from 85 peptide fragments identified in 19 patients with diastolic LV dysfunction and 19 controls.

References

## Table S1

List of polypeptides included in the HF1 classifier

<table>
<thead>
<tr>
<th>ID</th>
<th>Mass (Da)</th>
<th>CE Time (min)</th>
<th>Cases</th>
<th>Controls</th>
<th>R</th>
<th>P-value (Unadjusted)</th>
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<td>33.23</td>
<td>0 (0)</td>
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<tr>
<td>123106</td>
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<td>3.36 (0.05)</td>
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<td>1.91 (0.11)</td>
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<td>0.145</td>
<td>0.001</td>
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(continued on next page)
Table S1 (continued)

<table>
<thead>
<tr>
<th>ID</th>
<th>Mass (Da)</th>
<th>CE Time (min)</th>
<th>Cases MA (%)</th>
<th>Controls MA (%)</th>
<th>R</th>
<th>P-value (Unadjusted)</th>
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<td>9.266</td>
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</table>

ID, polypeptide identifier (SQL number); %, percentage of samples, in which the polypeptide could be detected; MA, mean signal amplitude of the polypeptides.

R was calculated as $\sum (\text{ln signal amplitude} \times \text{frequency/number of participants})$ in controls divided by $\sum (\text{ln signal amplitude} \times \text{frequency/number of participants})$ in cases. The polypeptides were ordered by ascending R. Published under CC BY-NC-ND license.
### Table S2
Echocardiographic measurements by quartiles of the HF1 distribution

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<th>Characteristic</th>
<th>Categories of the Urinary HF1 Biomarker</th>
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<td>Limits, Score</td>
<td>&lt;-1.623</td>
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<tr>
<td>Conventional echocardiography</td>
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</tr>
<tr>
<td>Left atrial volume index, mL/m²</td>
<td>24.6 ± 5.46</td>
</tr>
<tr>
<td>Left ventricular mass index, g/m²</td>
<td>89.6 ± 16.7</td>
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<tr>
<td>Doppler data</td>
<td></td>
</tr>
<tr>
<td>E peak, cm/s</td>
<td>70.8 ± 14.8</td>
</tr>
<tr>
<td>A peak, cm/s</td>
<td>56.4 ± 13.8</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.35 ± 0.50</td>
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<tr>
<td>e′ peak, cm/s</td>
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<tr>
<td>a′ peak, cm/s</td>
<td>9.08 ± 2.02</td>
</tr>
<tr>
<td>e′/a′ ratio</td>
<td>1.40 ± 0.71</td>
</tr>
<tr>
<td>E/e′ ratio</td>
<td>6.56 ± 1.81</td>
</tr>
</tbody>
</table>

*Significance of the difference with the adjacent lower quartile: P ≤ .05.
†P ≤ .01.
‡P ≤ .001.