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Left ventricular diastolic function in relation to the urinary proteome: A proof-of-concept study in a general population

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ABSTRACT

Background: In previous studies, we identified two urinary proteomic classifiers, termed HF1 and HF2, which discriminated subclinical diastolic left ventricular (LV) dysfunction from normal. HF1 and HF2 combine information from 85 and 671 urinary peptides, mainly up- or down-regulated collagen fragments. We sought to validate these classifiers in a population study.

Methods: In 745 people randomly recruited from a Flemish population (49.8 years; 51.3% women), we measured early and late diastolic peak velocities of mitral inflow (E and A) and mitral annular velocities (e' and a') by conventional and tissue Doppler echocardiography, and the urinary proteome by capillary electrophoresis coupled with mass spectrometry.

Results: In the analyses adjusted for sex, age, body mass index, blood pressure, heart rate, LV mass index and intake of medications, we expressed effect sizes per 1-SD increment in the classifiers. HF1 was associated with 0.204 cm/s lower e' peak velocity (95% confidence interval, 0.057–0.351; $p = 0.007$) and 0.145 higher E/e' ratio (0.023–0.268; $p = 0.020$), while HF2 was associated with a 0.174 higher E/e' ratio (0.046–0.302; $p = 0.008$). According to published definitions, 67 (9.0%) participants had impaired LV relaxation and 96 (12.9%) had elevated LV filling pressure. The odds of impaired relaxation associated with HF1 was 1.38 (1.01–1.88; $p = 0.043$) and that of increased LV filling pressure associated with HF2 was 1.38 (1.00–1.90; $p = 0.052$).

Conclusions: In a general population, the urinary proteome correlated with diastolic LV dysfunction, proving its utility for early diagnosis of this condition.

1. Introduction

Recent guidelines [1,2] describe heart failure (HF) as a complex clinical syndrome that results from any structural or functional impairment of ventricular filling or ejection of blood. HF may result from disorders of the pericardium, myocardium, endocardium, heart valves, or great vessels or from certain metabolic abnormalities, but most HF patients have symptoms due to impaired left ventricular (LV) myocardial function with or without preserved ejection fraction. Impaired LV function evolves from asymptomatic changes in cardiac structure (e.g. LV hypertrophy) and function (e.g. impaired relaxation) into clinically overt HF, disability and death. The 5-year mortality rate of symptomatic HF is approximately 60% [3]. Diastolic HF is characterised by slow LV relaxation, increased LV stiffness, increased interstitial deposition of collagen, and modified extracellular matrix proteins [4]. Diastolic HF accounts for 40–50% of all HF cases and has a prognosis as ominous as systolic HF [4]. In randomly recruited European population samples, the frequency of asymptomatic echocardiographically diagnosed diastolic LV dysfunction (early stage) is as high as 27% [5,6]. This constitutes a large pool of subjects at high risk of diastolic HF.

The pathogenesis underlying diastolic LV dysfunction might rest on atherosclerosis of the large epicardial or intramural coronary arteries [7,8]. More recently experts in the field advanced the hypothesis that endothelial dysfunction in the coronary microcirculation and a systemic pro-inflammatory state favour the development of LV hypertrophy, stiffening of cardiomyocytes and interstitial myocardial fibrosis [9,10]. Whatever the underlying mechanism, modification in the extracellular myocardial matrix and collagen turnover are hallmarks of diastolic LV dysfunction. In line with this concept, we identified in a preliminary

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case–control study 85 urinary peptides, mainly up- or down-regulated collagen fragments, that discriminated between 19 hypertensive patients with asymptomatic diastolic LV dysfunction and 19 controls [11]. With adjustments applied for multiple testing three urinary peptide biomarkers remained significant [11]. In an attempt to find ways to facilitate the diagnosis of asymptomatic diastolic LV dysfunction, we evaluated in a Flemish population sample the association of diastolic LV function, analysed as a continuous or categorical variable, with urinary proteomic biomarkers combined in a high-dimensional model (classifier).

2. Material and methods

2.1. Participants

The Ethics Committee of the University of Leuven approved the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO) [12,13]. Our study was designed to enrol a random population sample with families as the sampling unit. Recruitment started in 1985 [13]. The initial participation rate was 78.0%.

From May 2005 until May 2010, we mailed an invitation letter to 1208 former participants for a re-examination, including echocardiography. However, 153 former participants were unavailable for follow-up, because they had died ($n = 26$), because they had been institutionalised 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 for the re-examination was therefore 78.5%. We excluded from analysis 19 cases and 19 controls, because they had been selected to identify one of the multidimensional classifiers used in the current analyses [11]. Furthermore, we removed an additional 45 participants from analysis, because no urine sample was available for urinary proteomics ($n = 22$), because of atrial fibrillation ($n = 8$) or paced heart rhythm ($n = 3$), because their LV mass ($n = 6$) or diastolic LV function could not be reliably determined ($n = 6$). Thus, the number of participants included in the current cross-sectional analysis totalled 745.

2.2. Echocardiography

2.2.1. Data acquisition

One experienced physician (T.K.) did the ultrasound examination [5], using a Vivid7 Pro (GE Vingmed, Horten, Norway) interfaced with a 2.5- to 3.5-MHz phased-array probe. For off-line analysis, she recorded at least five heart cycles according to the recommendations of the American Society of Echocardiography [14]. M-mode echocardiograms of the LV were recorded from the parasternal long-axis view under control of the 2-dimensional image. The ultrasound beam was positioned just below the mitral valve at the level of the posterior tendinous chords. To record mitral and pulmonary vein (PV) flow velocities from the apical window, the observer positioned the Doppler sample volume at the mitral valve tips, in the right superior PV, and between the LV outflow and mitral inflow, respectively. From the apical window, the observer positioned a 5-mm Doppler sample at the septal, lateral, inferior and posterior sites of the mitral annulus to record low-velocity, high-intensity myocardial signals at a high frame rate (>190 frames per second), while ensuring parallel alignment of the ultrasound beam with the myocardial segment of interest.

2.2.2. Off-line analysis

One reader (T.K.) analysed the digitally stored images, averaging three heart cycles, using a workstation running EchoPac software, version 4.0.4 (GE Vingmed, Horten, Norway). LV internal diameter and interventricular septal and posterior wall thickness were measured at end-diastole from the 2-dimensionally guided M-mode tracing. When optimal orientation of M-mode ultrasound beam could not be obtained, the reader performed linear measurements on correctly oriented 2-dimensional images. End-diastolic LV dimensions were used to calculate LV mass by an anatomically validated formula [14]. Left atrial (LA) volume was calculated using the prolate-ellipsoid method from the LA dimensions in three orthogonal planes and indexed to body surface area [14]. From the transmitral flow signal, the reader determined peak early diastolic velocity (E), peak late diastolic velocity (A), the E/A ratio, and transmitral A flow duration. From the PV flow signal, she measured the duration of PV reversal flow during atrial systole (AR). From the TDI recordings, the reader measured peak early (e') and peak late (a') diastolic mitral annular velocities, and the e'/a' ratio at the four acquisition sites (septal, lateral, inferior, and posterior).

2.2.3. Reproducibility

Intra-observer reproducibility was the 2-SD interval about the mean of the relative differences across pairwise readings. As reported previously [5], the intra-observer reproducibility for the tissue Doppler peak velocities across the four sampling sites ranged from 4.48% to 5.34% for e' and from 3.96% to 4.52% for a'. For the LV internal end-diastolic diameter, reproducibility was 4.6% for LV wall thickness and 4.3% for LV mass [15].

2.2.4. Classification of diastolic LV function

For staging LV diastolic dysfunction, the mitral inflow and TDI velocities were combined, as previously described [5,6]. The first group included patients with an abnormally low age-specific transmitral E/A ratio indicative of impaired relaxation, but without evidence of increased LV filling pressures (E/e' ≤8.5). The second group had mildly-to-moderately elevated LV filling pressure (E/e' >8.5) and an E/A ratio within the normal age-specific range. Differences in durations between the transmitral A flow and the reverse PV flow during atrial systole (Ad < ARd + 10) and/or LA volume index (≥28 mL/m^2) were checked to confirm possible elevation of the LV filling pressures in group 2. The third group had an elevated E/e' ratio and an abnormally low age-specific E/A ratio (combined dysfunction).

2.3. Urinary proteomics

Participants collected 24-h urine samples within 1 week of the echocardiographic examinations. Using 24-h urine samples rather than spot urine samples minimises the small but detectable influence of food intake [16] during the day on the urinary proteome. Aliquots 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 NH4OH containing 0.02% SDS [17]. To remove higher molecular mass proteins, such as albumin and immunoglobulin G, the sample was ultra-filtered using Centrisart ultracentrifugation devices (20 kDa MWCO; Sartorius, Göttingen, Germany) at 3000 *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 H_2O (Roth, Germany) to decrease matrix effects by removing urea, electrolytes, and salts, and to enrich polypeptides. Finally, all samples were lyophilised, stored at 4 °C, and suspended in HPLC-grade H_2O shortly before CE–MS analyses [18].

CE–MS analyses were performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) on-line coupled to a micrOTOF MS (Bruker Daltonic, Bremen, Germany) [18]. 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 s, 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 [19].

Mass spectra were processed using MosaiquesVisu software, including peak picking, deconvolution and de-isotoping [20]. Migration time and peak intensity were normalised using internal polypeptide standards [21]. 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 [22]. The resulting peak list characterises each polypeptide by its molecular mass, normalised capillary electrophoresis migration time, and normalised signal intensity. All detected polypeptides were deposited, matched, and annotated in a Microsoft SQL database, allowing further analysis and comparison of multiple patient groups.

Peptide fragments identified in previous studies 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 two high-dimensional classifiers. As published previously [11], HF1 combined information from 85 peptide fragments identified in 19 patients with diastolic LV dysfunction and 19 controls. To generate the HF2 classifier, all urinary proteomic datasets from cases available in the Mosaiques database [22] were combined and compared with data from sex- and age-matched controls. Cases were 98 patients with LV diastolic dysfunction recruited from our population [11] ($n = 35$) or admitted to the hospital because of overt HF ($n = 63$). The patients with overt HF were all on multiple drugs, included 49.2% women and were 67.1 ± 9.9 years old. The underlying cause of HF was ischaemic cardiomyopathy (50.8%), dilated cardiomyopathy (28.6%), hypertrophic cardiomyopathy (1.6%), valvular heart disease (1.6%) or unspecified (17.4%). Comparing cases with controls identified 710 potential biomarkers, based on a *p*-value of less than 0.05 with adjustment for multiple testing applied. Using a take-one-out procedure [23] to remove potential biomarkers that are of no apparent value, the number of biomarkers was reduced to 671. A MosaiquesVisu software based classifier including these 671 urinary peptides was developed, using all 196 (98 cases and 98 controls) datasets. Upon complete take-one-out cross-validation, in the training dataset, the classifier had 88.7% accuracy, 87.8% sensitivity, and 89.58% specificity. Full information of the polypeptides making up the two classifiers (Tables S1 and S2) and on the polypeptides with known amino-acid sequence (Tables S3 and S4) is available in the Supplementary material online. A subset of 671 participants had plasma NT-proBNP measured by a competitive enzyme immunoassay developed for research purposes only use (Biomedica Gruppe, Vienna, Austria) [24].

2.4. Other measurements

At the examination centre, nurses administered a questionnaire to collect detailed information on each participant's medical history, smoking and drinking habits, and intake of medications. The conventional blood pressure was the average of five consecutive auscultatory readings obtained with the subject in the seated position. Hypertension was a blood pressure of at least 140 mmHg systolic or 90 mmHg diastolic or use of antihypertensive drugs. Body mass index was weight in kilogrammes divided by the square of height in metres. Participants fasted for 6 h or longer prior to venepuncture. Venous blood samples were analysed for glucose, creatinine, total and high-density (HDL) cholesterol, and γ-glutamyltransferase as index of alcohol intake. We computed low-density (LDL) cholesterol, using Friedewald's formula [25]. We applied the Modification of Diet in Renal Disease Study Group equation (MDRD) to estimate the glomerular filtration rate (eGFR) from sex, age, and serum creatinine [26]. Diabetes mellitus was a self-reported diagnosis, a fasting glucose level of at least 126 mg/dL, or use of antidiabetic agents [27]. In 671 participants, NT-proBNP was measured in plasma by a competitive enzyme immunoassay (EIA) for research use (Biomedica Gruppe, Vienna, Austria).

Participants also collected an exactly timed 24-h urine sample for the measurement of micro-albuminuria. Micro-albuminuria was a 24-h urinary excretion ranging from 30 to 300 mg and macro-albuminuria a 24-h excretion exceeding 300 mg.

2.5. Statistical analysis

For database management and statistical analysis, we used the SAS system, version 9.3 (SAS Institute Inc., Cary, NC). Significance was a two-tailed α-level of 0.05 or less. Means were compared using the large-sample z-test or ANOVA and proportions by Fisher's exact test. The distribution of γ-glutamyltransferase was normalised by a logarithmic transformation. Our statistical methods also included multivariable-adjusted linear and logistic regression analysis with as dependent variables LV mass, the indexes reflecting diastolic LV function or the categories of diastolic LV dysfunction. The covariables accounted for in all analyses were sex, age, body mass index, mean arterial pressure, heart rate, LV mass index, treatment with inhibitors of the renin system (angiotensin-converting enzyme inhibitors or angiotensin II type-1 receptor blockers), and use of β-blockers. In sensitivity analyses, models were additionally adjusted for blood glucose, serum creatinine and γ-glutamyltransferase as index of alcohol intake. To maximise the discriminatory accuracy of HF1 and HF2, we maximised Younden's index (sensitivity + specificity − 1) in unadjusted logistic models. Finally, we assessed the added capacity of the urinary proteomic biomarkers to differentiate normal from abnormal diastolic LV function, using the integrated discrimination improvement (IDI) and the net reclassification improvement (NRI) [28].

2.6. Role of the funding source

The funding source had no role in the study design, data extraction, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had the responsibility for the decision to submit for publication.

3. Results

3.1. General characteristics of participants

Women ($n = 382$) and men ($n = 363$) had similar age (mean, 49.8 years; range, 18 to 89 years). The study sample included 309 (41.5%) hypertensive patients of whom 185 (59.9%) were on antihypertensive drug treatment, and 9 participants (1.2%) had diabetes mellitus. Among the 185 patients on treatment with antihypertensive drugs, 64 (34.6%) used inhibitors of the renin system (angiotensin converting enzyme inhibitors or angiotensin II type-1 receptor blockers), and 109 (58.9%) were on treatment with β-blockers. Of 382 women and 363 men, 74 (19.4%) women and 74 (20.4%) men were smokers, and 214 (56.0%) women and 301 (82.9%) men reported intake of alcohol. In smokers, median tobacco use was 15 cigarettes per day (interquartile range, 7 to 20 cigarettes per day). In drinkers, the median alcohol consumption was 10 g per day (interquartile range, 4 to 17 g per day). Only one participant had a history of HF. Thirty patients had mild to moderate valvular heart disease, including, aortic stenosis ($n = 3$), aortic regurgitation ($n = 6$), mitral regurgitation ($n = 10$), tricuspid regurgitation ($n = 2$), or a combinations thereof. One participant had Marfan syndrome. The prevalence of micro- and macro-albuminuria in our study population was 26 (3.5%) and 3 (0.4%), respectively.

3.2. Analyses across categories of HF1 and HF2

Fig. 1 shows the distributions of HF1 and HF2. Table 1 lists the characteristics of participants by fourths of the distribution of HF1. Age, body mass index, blood pressure, the prevalence of hypertension and use of antihypertensive drugs, LDL cholesterol, blood glucose, serum creatinine and γ-glutamyltransferase increased ($p \leq 0.049$) with higher HF1 category, whereas the opposite was the case for HDL cholesterol and eGFR ($p \leq 0.049$). In the subset 671 participants who had NT-proBNP measured, the geometric mean level was 203.8 pmol/L (interquartile range, 140.1 to 287.3 pmol/L). The NT-proBNP levels did not increase with higher categories of HF1 (Table 1) and HF2 (Table S5). HF2 ($r = 0.10$; $p = 0.009$), but not HF1 ($r = 0.02$; $p = 0.65$) correlated with the logarithmically transformed NT-proBNP level.

Table 2 shows that LA volume, LA volume index, LV mass, LV mass index, deceleration time, isovolumetric relaxation time, A and a' peak velocities, and the E/e' ratio increased ($p \leq 0.0007$) with higher HF1 category, whereas the opposite was the case ($p < 0.0001$) for the E and e' peak velocities and the E/A and e'/a' ratios. Findings were similar across fourths of the HF2 distribution for the characteristics of the participants (Table S5) as well as for the echocardiographic measurements (Table S6). Additional information on the risk factor scores and comorbidities by fourths of the distributions of HF1 and HF2 are provided in Table S7.

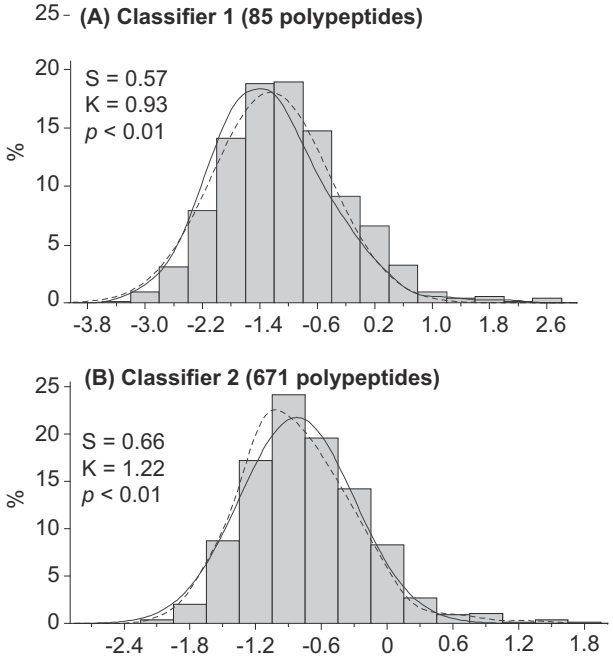

Fig. 1. Distribution of the urinary multi-dimensional biomarkers HF1 (A) and HF2 (B) in 745 participants. S and K are the coefficients of skewness and kurtosis, respectively. The *p*-value is for the Kolmogorov–Smirnov test and indicates departure of the actually observed distribution (full line) from normality (dotted line).

3.3. Multivariable-adjusted analyses of continuous echocardiographic measures

With adjustments applied for sex, age, body mass index, mean arterial pressure, heart rate, LV mass index, treatment with inhibitors of the renin system, and use of β-blockers, as shown in Table 3, a 1-SD increment in HF1 was associated with a 0.908 mL decrease in LA volume, a 0.473 mL/m^2 lower LA volume index, a 0.204 cm/s lower e' peak velocity ($p = 0.007$), a 2.512 ($p = 0.049$) longer deceleration time and a 0.145 higher E/e' ratio ($p = 0.020$). With similar adjustments, a 1-SD increment in HF2 was associated with a 3.971 ($p = 0.003$) longer deceleration time, a 0.174 higher E/e' ratio ($p = 0.008$), a 0.025 higher E/A ratio ($p = 0.037$), and a 0.152 cm/s lower a' peak velocity ($p = 0.008$). The association between the E/A ratio and HF2 was driven by patients with increased LV filling pressure. After removal of these 96 patients, the association size weakened to 0.014 (95% confidence interval, −0.013 to 0.041; $p = 0.31$). LV mass and the other Doppler indexes were not significantly associated with HF1 ($0.091 \leq p \leq 0.93$) or HF2 ($0.22 \leq p \leq 0.82$). As shown in Table S8, analyses of the Doppler measurements additionally adjusted for blood glucose, serum creatinine and γ-glutamyltransferase were confirmatory.

3.4. Multivariable-adjusted odds ratios for of diastolic LV dysfunction

According to the definitions given in the methods and in previous publications [5,6] 67 (9.0%) participants had impaired LV relaxation, 80 (10.7%) had elevated LV filling pressure, and 16 (2.1%) had a combined dysfunction. To allow a multivariable analysis, we pooled

Table 1
Patient characteristics by fourths of the distribution of HF1.

Characteristic	Categories of the urinary HF1 biomarker				*P*
Limits, score	<−1.639	−1.639 to −1.078	−1.077 to −0.489	≥−0.488	
Number of subjects (%)					
All patients in category	187	187	185	186	
Women	96 (51.3)	96 (51.3)	101 (54.6)	89 (47.9)	0.64
Smokers	47 (25.1)	37 (19.8)	34 (18.4)	30 (16.1)	0.16
Drinking alcohol	135 (72.2)	131 (70.1)	123 (66.5)	126 (67.7)	0.64
Hypertension	53 (28.3)	62 (33.2)	78 (42.2)	116 (62.4)§	<0.0001
Antihypertensive treatment	25 (13.4)	29 (15.5)	44 (23.8)*	87 (46.8) §	<0.0001
Diabetes mellitus	3 (1.6)	0	1 (0.5)	5 (2.7)	0.084
Mean (SD) of characteristic					
Age, years	44.4 ± 14.5	47.3 ± 15.3	50.9 ± 14.5*	56.7 ± 14.8‡	<0.0001
Body mass index, kg/m^2	25.4 ± 3.7	25.8 ± 3.7	26.5 ± 4.4	27.8 ± 4.7†	<0.0001
Office blood pressure					
Systolic pressure, mmHg	125.6 ± 14.7	127.7 ± 18.6	129.3 ± 17.9	132.5 ± 17.3	0.001
Diastolic pressure, mmHg	78.7 ± 9.1	78.7 ± 10.2	80.4 ± 9.2	80.8 ± 9.5	0.049
Mean arterial pressure, mmHg	94.3 ± 9.8	95.0 ± 11.3	96.7 ± 10.8	98.0 ± 10.5	0.003
Heart rate, beats per minute	61.1 ± 9.2	60.4 ± 9.6	60.4 ± 9.7	61.2 ± 10.7	0.79
Biochemical data					
Total cholesterol, mmol/L	5.09 ± 0.96	5.27 ± 0.97	5.30 ± 0.95	5.29 ± 1.01	0.11
HDL cholesterol, mmol/L	1.46 ± 0.35	1.44 ± 0.34	1.44 ± 0.37	1.37 ± 0.35*	0.049
LDL cholesterol, mmol/L	3.05 ± 0.87	3.23 ± 0.85*	3.23 ± 0.84	3.24 ± 0.87	0.042
Blood glucose, mmol/L	4.86 ± 0.55	4.82 ± 0.50	4.95 ± 0.68*	5.15 ± 1.23	0.0003
Serum creatinine, μmol/L	81.7 ± 13.2	82.2 ± 13.3	83.2 ± 13.7	87.1 ± 20.4*	0.003
eGFR, mL/min/1.73 m^2	84.4 ± 14.8	83.5 ± 19.0	79.6 ± 14.4*	76.5 ± 16.7	<0.0001
γ-glutamyltransferase, units/L	21 (12–37)	22 (12–59)	23 (11–48)	26 (13–61)*	0.003
NT-proBNP, pmol/L	211 (109–424)	193 (87–399)	211 (92–424)	200 (100–399)	0.42

Abbreviations: eGFR, estimated glomerular filtration rate calculated according to the MDRD formula, as described in reference 5510. Office blood pressure was the average of five consecutive readings. Heart rate was determined during the echocardiographic examination. Hypertension was an office blood pressure of ≥140 mmHg systolic, or ≥90 mmHg diastolic, or use on antihypertensive drugs. For γ-glutamyltransferase and NT-proBNP, values are geometric mean (interquartile range). NT-proBNP was measured in 167, 167, 166 and 171 participants of the 1st, 2nd, 3rd and 4th quartile, respectively (671 in total). *P* values denote the significance of the differences in prevalence rates or means across fourths of the HF1 distribution. Significance of the difference with the adjacent lower fourth: * $p \leq 0.05$; † $p \leq 0.01$; ‡ $p \leq 0.001$; § $p < 0.0001$.

patients with elevated LV filling pressure with the small group with combined LV dysfunction. With adjustments applied for sex, age, body mass index, mean arterial pressure, heart rate, LV mass index, treatment with inhibitors of the renin system, and use of β-blockers, as shown in Table 4 and Fig. S1, a 1-SD increment in HF1 was associated with a 38% higher risk of impaired relaxation ($p = 0.043$), but not with a significantly elevated risk of having an elevated LV filling pressure (odds ratio, 1.20; $p = 0.24$). A 1-SD increment in HF2 was weakly associated with a 38% higher risk of increased LV filling pressure ($p = 0.052$), but not with impaired relaxation (odds ratio, 1.24; $p = 0.22$). As shown in Table S9, analyses additionally adjusted for blood glucose, serum creatinine and γ-glutamyltransferase were confirmatory.

3.5. Improvement of diagnostic accuracy

By maximizing Younden's index, we determined optimal thresholds for HF1 and HF2 to differentiate normal from abnormal left diastolic LV function. Sensitivity of the optimised thresholds ranged from 65.6% to 93.8% and specificity from 31.1% to 66.3% (Table S10). For detecting any form of diastolic LV dysfunction, either impaired relaxation, increased filling pressure or both (Table S11), both HF1 and HF2 significantly ($p \leq 0.003$) enhanced NRI, but not IDI ($p \geq 0.12$) except for HF1 in the diagnosis of increased filling pressure ($p = 0.032$).

4. Discussion

The key finding of our study was that in a general population diastolic LV function correlated with multidimensional urinary proteomic classifiers. Our current findings extent those of a previously published case–control study [11]. In 19 asymptomatic hypertensive patients and 19 matched controls, we identified a set of 85 urinary polypeptides that discriminated diastolic LV dysfunction from normal LV function [11]. A subsequent replication study in 16 hypertensive patients and 16 controls confirmed the diagnostic accuracy of the set urinary peptides [11]. However, Redfield and colleagues demonstrated that among people with moderate or severe diastolic or systolic dysfunction, less than half had recognised congestive heart failure [29]. In multivariable-adjusted analyses, both mild and moderate or severe diastolic LV dysfunction predicted all-cause mortality over a median follow-up of 3.5 years [29]. The Olmsted County study [29], taken together with our current observations, suggest that the urinary proteome is a harbinger of clinical manifestations occurring later during the course of the disease.

Defining diagnostic thresholds for conditions, such as diastolic LV dysfunction, should be based on a randomly selected non-institutionalised sample of the general population [30,31]. To classify diastolic LV function, we first selected a healthy subsample from a Flemish population without history of cardiovascular disease and with low risk cardiovascular profile. In this reference group we determined age-specific cut-off points for the indexes of diastolic LV function, including E/e', E/A ratio, left atrial volume indexed to body surface area, and Δ(Ad–ARd). Next, we demonstrated that these indexes were consistent and reproducible in FLEMENGHO [5] and in population cohorts [6] enrolled in the European Project on Genes in Hypertension (EPOGH). For instance, the 97.5th percentiles of E/e' ratio in the FLEMENGHO [5] and EPOGH [6] reference groups were 8.6 and 8.5, respectively. Moreover, these E/e' thresholds were in keeping with the results of invasive studies that showed that a E/e' ratio below 8 is an accurate indicator of a normal LV filling pressure [32]. In the absence of an outcome-driven age-specific diagnostic reference frame, averaging the 2.5 and 97.5 percentiles for the E/A ratio in subjects free from cardiovascular diseases included FLEMENGHO [5] and EPOGH [6], and rounding the resulting boundaries to the closest integer value, produced working definitions of a normal E/A ratio. The lower boundaries of the age-specific thresholds for the E/A ratio decreased approximately by 0.10 per decade of age [5,6]. Absolute values of systolic and diastolic PV flow velocity and their ratio depend not only on diastolic LV properties, but also on

Table 2
Echocardiographic measurements by fourths of the distribution of HF1.

Characteristic	Categories of the urinary HF1 biomarker			
Limits, score	<−1.639	−1.639 to −1.078	−1.077 to −0.489	≥−0.488
Conventional echocardiography				
Left atrial volume, mL	40.0 ± 11.4	42.1 ± 13.3	43.9 ± 14.8	44.8 ± 14.5
Left atrial volume index, mL/m^2	21.6 ± 5.42	22.6 ± 6.28	23.2 ± 6.67	23.8 ± 6.83
Left ventricular mass, g	160.5 ± 43.5	169.4 ± 47.4	172.8 ± 50.7	183.6 ± 50.9*
Left ventricular mass index, g/m^2	86.5 ± 18.8	91.0 ± 20.5*	91.7 ± 22.1	97.8 ± 23.5†
Doppler data				
Deceleration time, ms	160.6 ± 30.6	158.3 ± 31.9	166.8 ± 33.9*	176.9 ± 43.2*
Isovolumetric relaxation time, ms	94.4 ± 14.6	96.6 ± 17.0	96.7 ± 14.1	100.3 ± 16.6*
E peak, cm/s	78.4 ± 14.9	78.3 ± 15.3	75.3 ± 16.5	71.2 ± 16.3*
A peak, cm/s	59.0 ± 16.0	61.2 ± 17.8	66.3 ± 17.0†	68.9 ± 15.8
E/A ratio	1.44 ± 0.51	1.39 ± 0.50	1.22 ± 0.45‡	1.10 ± 0.40†
e' peak, cm/s	13.2 ± 3.65	12.2 ± 3.49†	11.2 ± 3.29†	10.0 ± 3.25‡
a' peak, cm/s	9.51 ± 2.22	9.88 ± 2.19	10.2 ± 1.98	10.5 ± 1.92
e'/a' ratio	1.56 ± 0.80	1.37 ± 0.67*	1.19 ± 0.52†	1.02 ± 0.47†
E/e' ratio	6.25 ± 1.60	6.88 ± 2.19†	7.08 ± 1.99	7.59 ± 2.23*

All ANOVA p values for differences in means across fourths of the HF1 distribution were significant (≤0.0007). Significance of the difference with the adjacent lower fourth: * $p \le 0.05$; † $p \le 0.01$; ‡ $p \le 0.001$.

other factors, such as mitral regurgitation, younger age, LV systolic function, etc. [33]. These confounders might limit the use of PV flow velocity in the assessment of LV diastolic dysfunction, particularly, in general population [33]. On the other hand, we measured the difference in duration between the mitral A flow and AR. Invasive studies [32,34] demonstrated that a difference between the A wave and AR duration of >0 ms is associated with a LV end-diastolic pressure of 20 mmHg or greater with high sensitivity (82%) and specificity (92%). In our current study, we checked differences in durations between the transmitral A flow and the reverse PV flow during atrial systole (Ad < ARd + 10) and/or LA volume index (≥28 ml/m^2) to confirm possible elevation of the LV filling pressure.

In our current study, the E/e' ratio correlated positively with both HF1 and HF2. These observations carry prognostic information. Indeed, studies in patients with HF [35–37] or hypertension [38] demonstrated that high E/e' predicted cardiac mortality and re-hospitalisation for HF [35–37] or the risk of a cardiac event [38]. In the three HF studies [35–37], the E/e' ratio was the only [35] or a strong [36,37] predictor of the primary endpoint. Furthermore, a substudy to the Anglo-Scandinavian Outcomes Trial (ASCOT) [38] involved 980 high-risk hypertensive patients, free of cardiac disease at baseline and followed up for a median of 4.2 years. In multivariable-adjusted Cox-proportional hazards models, a unit rise in the E/e' ratio was associated with a 17% increment in risk of a cardiac event (95% confidence interval, 1.05–1.29; $p = 0.003$) [38].

In the present study, HF1 was not only associated with E/e', but with e' as well. In a Chinese cohort of 174 hypertensive patients and 78 age-matched controls, 19 patients (7.5%) experienced a fatal cardiac event during 1.6 years of follow-up [39]. In adjusted analyses, e' was among the strong predictors of cardiac mortality [39]. The positive association of HF2 with the transmitral E/A ratio and the inverse correlation of HF2 with a' peak mitral annular velocity at first sight looks counter-intuitive. However, Redfield and colleagues [29] demonstrated that in patients with severe diastolic LV dysfunction the transmitral A peak decreased resulting in a higher E/A ratio (so called pseudo-normalisation) and that the a' peak was lower than in subjects with normal diastolic LV function. To ensure that our interpretation of the positive association between the transmitral E/A ratio and HF2 was correct, we removed the 96 patients with increased LV pressure from analysis. This weakened the latter association to a non-significant level. The above reports [29,35–39] combined with the results of our current study highlight that urinary proteomic biomarkers, such as HF1 and HF2, might predict prognosis even in a population setting. Proving this hypothesis in the follow-up of our FLEMENGHO cohort is therefore a research priority.

Table 3
Multivariable-adjusted associations of echocardiographic measurements with 1-SD increases in HF1 and HF2.

Characteristic	Associations with HF1		Associations with HF2	
	Association size (95% confidence interval)	p	Association size (95% confidence interval)	p
Conventional echocardiography				
Left atrial volume, mL	−0.908 (−1.651 to −0.165)	0.017	0.643 (−1.425 to 0.138)	0.11
Left atrial volume index, mL/m^2	−0.473 (−0.850 to −0.096)	0.014	−0.284 (−0.681 to 0.113)	0.16
Left ventricular mass, g	0.118 (−2.568 to 2.805)	0.93	0.481 (−2.326 to 3.289)	0.74
Left ventricular mass index, g/m^2	0.697 (−0.662 to 2.056)	0.31	0.879 (−0.540 to 2.298)	0.22
Doppler data				
Deceleration time, ms	2.512 (0.013 to 5.012)	0.049	3.971 (1.367 to 6.575)	0.003
Isovolumetric relaxation time, ms	−0.179 (−1.234 to 0.877)	0.74	−0.687 (−1.791 to 0.416)	0.22
E peak, cm/s	−0.200 (−1.280 to 0.881)	0.72	0.603 (−0.525 to 1.732)	0.29
A peak, cm/s	−0.044 (−0.924 to 0.836)	0.92	−0.106 (−1.025 to 0.813)	0.82
E/A ratio	−0.004 (−0.026 to 0.019)	0.74	0.025 (0.002 to 0.049)	0.037
e' peak, cm/s	−0.204 (−0.351 to −0.057)	0.007	−0.082 (−0.236 to 0.072)	0.30
a' peak, cm/s	−0.104 (−0.224 to 0.017)	0.091	−0.152 (−0.278 to −0.026)	0.018
e'/a' ratio	−0.018 (−0.047 to 0.010)	0.20	0.004 (−0.025 to 0.034)	0.77
E/e' ratio	0.145 (0.023 to 0.268)	0.020	0.174 (0.046 to 0.302)	0.008

Association were expressed for a 1-SD increase in the multidimensional classifiers HF1 and HF2. All association sizes were adjusted for sex, age, mean arterial pressure, heart rate, treatment with inhibitors of the renin system (angiotensin-converting enzyme inhibitors or angiotensin II type-1 receptor blockers), and use of β-blockers. Left atrial volume and left ventricular mass were additionally adjusted for body mass index and the Doppler data for body mass index and left ventricular mass index.

Table 4
Multivariable-adjusted odds ratios for a 1-SD increase in HF1 or HF2.

Characteristic	Associations with HF1		Associations with HF2	
	Odds ratio(95% confidence interval)	*p*	Odds ratio(95% confidence interval)	*p*
Impaired relaxation ($n = 67$)	1.38 (1.01 to 1.88)	0.043	1.24 (0.88 to 1.74)	0.22
Increased filling pressure ($n = 96$)	1.20 (0.88 to 1.63)	0.24	1.38 (1.00 to 1.90)	0.052

Impaired relaxation refers to patients with an abnormally low age-specific transmitral E/A ratio indicative without evidence of increased LV filling pressures (E/e' ≤8.5). Increased filling pressure refers to patients with elevated LV filling pressure (E/e' >8.5) and an E/A ratio within or below the normal age-specific range. Association were expressed for a 1-SD increase in the multidimensional classifiers HF1 and HF2. All association sizes were adjusted for sex, age, body mass index, mean arterial pressure, heart rate, left ventricular mass index, treatment with inhibitors of the renin system (angiotensin-converting enzyme inhibitors or angiotensin II type-1 receptor blockers), and use of β-blockers.

Moreover, randomised clinical trials in patients with diastolic LV dysfunction should establish that the urinary proteome changes in parallel with the echocardiographic findings and clinical outcomes.

Redfield and coworkers categorised diastolic LV function as ranging from normal to severe [29]. Mild diastolic dysfunction was impaired relaxation without evidence of increased filling pressures; moderate, was impaired relaxation associated with moderate elevation of filling pressures or pseudo-normal filling; and severe, was advanced reduction in compliance with restrictive LV filling. In the present study, a 1-SD increment in HF1 was associated with a 38% higher risk of impaired relaxation, but not with a significantly elevated risk of having an elevated LV filling pressure, whereas a 1-SD increment in HF2 was associated with a 38% higher risk of increased LV filling pressure, but not with impaired relaxation. These observations probably reflect the study populations, from which the classifiers were derived. HF1 rests on a case–control study including predominantly asymptomatic mild diastolic LV dysfunction, whereas the cases from which HF2 was derived predominantly consisted of patients hospitalised for overt HF.

In addition to HF1 and HF2, other correlates might help in screening for diastolic LV dysfunction, including albuminuria [40], circulating NT-proBNP [5], and the renal resistance index [41]. However, in our participants randomly recruited from a general population the prevalence of albuminuria was too low to be useful as a screening instrument. Among asymptomatic participants, there is large overlap in the NT-proBNP levels between categories of diastolic LV function. We did not measure the renal resistance index, which is a measure of renal blood flow obtained by Doppler ultrasonography. Ciccone and coworkers recently demonstrated in 250 out-patients with congestive heart failure in stable condition that the resistance index predicted rehospitalisation, progression to cardiac transplantation or heart failure death [41]. In the further follow-up of our participants we are therefore measuring the resistance index to test its prognostic value in relation to HF in largely asymptomatic subjects recruited from a general population.

Under physiological conditions, the urinary proteome originates for about 70% from the kidney and the urinary tract, while 30% is derived from plasma [42]. Approximately 60% of the total mass of urinary peptides and proteins consist of collagen fragments [22]. In our case–control study, we demonstrated that most of the markers originated from collagen [11]. Similarly, of the urinary peptides with known amino-acid sequence that were included in HF2, 68.9% were collagen fragments. The cardiac extracellular matrix predominantly consist of fibrillar collagen type I (85%) and type III (11%). In patients with HF, the balance between collagen synthesis and degradation is disturbed [43]. Diastolic LV dysfunction and HF associated with hypertension are characterised by increased interstitial deposition and cross-linking of type I collagen, a process that leads to LV stiffening [43]. However, our current study cannot prove the cardiac origin of the urinary collagen fragments that contribute to HF1 and HF2. For this reason, we are currently pursuing two research avenues. First, we will run proteomics on biopsies taken from explanted (diseased) and implanted (healthy) hearts during cardiac transplantation surgery in an attempt to prove that the urinary and tissue proteomic signatures are similar. Second, we will link the urinary proteomic collagen fragments with circulating biomarkers of collagen synthesis and degradation [44]. Circulating biomarkers of interest are the inhibitors of metalloproteinases that degrade collagen, such as TIMP-1 and TIMP-4 [45]. The expression of these inhibitors is tissue specific [45]. Linking the urinary proteome with these circulating tissue-specific inhibitors will therefore help in differentiating the cardiac vs. renal origin of the urinary collagen fragments.

5. Study limitations

The present study must be interpreted within the context of its limitations. Foremost, our findings arose from a cross-sectional analysis. Whether or not, the urinary proteomic biomarkers can predict the course over time of diastolic LV dysfunction and associated cardiovascular complications remains to be proven in longitudinal studies and in randomised clinical trials. However, the Olmsted County study [29] in combination with our current findings suggest that the urinary proteome might well be an early marker of incumbent disease. Second, HF1 and HF2 still await validation against invasive measures of diastolic LV function, such as the time constant of isovolumetric relaxation (τ). However, several studies showed that τ as assessed by transthoracic Doppler underestimates the invasively measured τ, but that this slight underestimation is correctable by accounting for left atrial or capillary wedge pressure [46,47]. Third, 228 of 973 people (23.4%) declined participation in our current study. However, participants and non-participants had similar distributions of female sex (51.3% vs. 51.3%; $p = 0.99$), age (49.8 vs. 47.3 years; $p = 0.09$), and previous cardiovascular disease (4.4% vs. 4.8%; $p = 0.78$). Fourth, we did not perform a Valsalva manoeuvre. However, major limitations of the Valsalva manoeuvre are that not every person can perform it adequately and that it is difficult to standardize. Moreover, its clinical value in distinguishing normal from pseudo-normal mitral inflow substantially diminished since TDI echocardiography of the mitral annulus allows a more precise assessment of LV relaxation and estimation of LV filling pressures. As proposed by experts [48], a Valsalva manoeuvre is only meaningful if the echocardiographic assessment of diastolic LV function leaves room for doubt, which was not the case in our participants. Finally, before being clinically applicable our results need replication in other population studies and in people of non-white ethnicity. On the other hand, both HF1 and HF2 significantly enhanced NRI differentiating normal from abnormal diastolic LV function, but did not substantially improve IDI. Our observations on IDI and NRI are not contradictory. NRI is a categorical measure. It measures in how many cases the probability of a positive diagnosis increases when adding the biomarker to the model and vice versa in non-cases. IDI is a continuous measure. It measures how much the probability of a positive diagnosis in cases increases by adding the biomarker to the model, and vice versa in the non-cases. Both metrics provide complimentary information. Adding a biomarker to the model might increase the probability of identifying a case, which means an increase in NRI, but perhaps only to a limited extend, as reflected by IDI.

6. Conclusions

Our current study extends the findings of a previous case–control study [11] to a larger population-based sample and suggest that the urinary proteome is useful for early diagnosis of diastolic LV dysfunction. However, only prospective studies showing that the urinary proteome predicts progression of diastolic LV dysfunction and randomised clinical trials demonstrating that the urinary proteome changes in parallel with the response to treatment can turn this screening tool into a clinically applicable modality. Having this research goal materialised would be a major step forward in view of the high prevalence of diastolic LV dysfunction [5,6] and the associated risk of progression to overt HF [29].

Conflict of interest

T Koeck, P Zürbig and H Mischak are employees of Mosaiques-Diagnostics GmbH. None of the other authors declares a conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2014.07.014.

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