International Journal of Cardiology 176 (2014) 158–165

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

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

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ARTICLE INFO

Article history:
Received 2 April 2014
Received in revised form 6 May 2014
Accepted 5 July 2014
Available online 12 July 2014

Keywords:
Diastolic dysfunction
Urinary proteomics
Population science

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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http://dx.doi.org/10.1016/j.ijcard.2014.07.014

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).