

Supplementary Table 1

Diagnostic accuracy of B-lines and other echocardiographic or bio-humoral tools in the diagnosis of HFpEF (Exercise stress echocardiography cohort and cycle ergometer test cohort)

Variable	Exercise stress echocardiography cohort				Cycle ergometer test cohort			
	Sensitivity	Specificity	Negative predictive value	Positive predictive value	Sensitivity	Specificity	Negative predictive value	Positive predictive value
Echocardiography								
Average E/e' (rest) ≥9	30.3 (20.2 - 41.9)	80.0 (64.4 - 90.9)	37.6 (27.4 - 48.8)	74.2 (55.4 - 88.1)	//////	//////	//////	//////
Average E/e' (exercise) ≥15	30.3 (20.2 - 41.9)	100 (91.2 - 100)	43.0 (32.8 - 53.7)	100.0 (85.2 - 100.0)	//////	//////	//////	//////
Natriuretic peptides								
BNP ≥35 pg/ml	46.1 (34.5 - 57.9)	97.5 (86.8 - 99.9)	48.8 (37.4 - 60.2)	97.2 (85.5 - 99.9)	//////	//////	//////	//////
HFpEF scores								
H2FPEF score ≥6	10.5 (4.7 - 19.7)	100 (91.2 - 100)	37.0 (27.9 - 46.9)	100 (63.1 - 100)	//////	//////	//////	//////
HFA-PEFF score (rest) ≥5	30.3 (20.2 - 41.9)	100 (91.2 - 100)	43.0 (32.8 - 53.7)	100 (85.2 - 100)	//////	//////	//////	//////
HFA-PEFF score (exercise) ≥5	47.4 (35.8 - 59.2)	100 (91.2 - 100)	50.0 (38.6 - 61.4)	100 (90.3 - 100)	//////	//////	//////	//////
Lung ultrasound								
Peak B-lines > 1	100 (95.3 - 100)	27.5 (14.6 - 43.9)	100 (71.5 - 100)	72.4 (62.8 - 80.7)	55.6 (35.3 - 74.5)	85.2 (66.3 - 95.8)	65.7 (47.8 - 80.9)	78.9 (54.4 - 93.9)
Peak B-lines > 2	98.7 (92.9 - 100)	57.5 (40.9 - 73.0)	95.8 (78.9 - 99.9)	81.5 (72.1 - 88.9)	51.9 (31.9 - 71.3)	85.2 (66.3 - 95.8)	63.9 (46.2 - 79.2)	77.8 (52.4 - 93.6)
Peak B-lines > 3	98.7 (92.9 - 100)	77.5 (61.5 - 89.2)	96.9 (83.8 - 99.9)	89.3 (80.6 - 95.0)	51.9 (31.9 - 71.3)	88.9 (70.8 - 97.6)	64.9 (47.5 - 79.8)	82.4 (56.6 - 96.2)
Peak B-lines > 4	94.7 (87.1 - 98.5)	90.0 (76.3 - 97.2)	90.0 (76.3 - 97.2)	94.7 (87.1 - 98.5)	44.4 (25.5 - 64.7)	88.9 (70.8 - 97.6)	61.5 (44.6 - 76.6)	80.0 (51.9 - 95.7)
Peak B-lines > 5	93.4 (85.3 - 97.8)	97.5 (86.8 - 99.9)	88.6 (75.4 - 96.2)	98.6 (92.5 - 100)	33.3 (16.5 - 54.0)	96.3 (81.0 - 99.9)	59.1 (43.2 - 73.7)	90.0 (55.5 - 99.7)
Peak B-lines < 2	0.0 (0.0 - 4.7)	72.5 (56.1 - 85.4)	27.6 (19.3 - 37.2)	0.0 (0.0 - 28.5)	44.4 (25.5 - 64.7)	14.8 (4.2 - 33.7)	21.1 (6.1 - 45.6)	34.3 (19.1 - 52.2)
Peak B-lines < 3	1.3 (0.0 - 7.1)	42.5 (27.0 - 59.1)	18.5 (11.1 - 27.9)	4.2 (0.1 - 21.1)	48.1 (28.7 - 68.1)	14.8 (4.2 - 33.7)	22.2 (6.4 - 47.6)	36.1 (20.8 - 53.8)
Peak B-lines < 4	1.3 (0.0 - 7.1)	22.5 (10.8 - 38.5)	10.7 (5.0 - 19.4)	3.1 (0.1 - 16.2)	48.1 (28.7 - 68.1)	11.1 (2.4 - 29.2)	17.6 (3.8 - 43.4)	35.1 (20.2 - 52.5)
Change B-lines > 1	100 (95.3 - 100)	32.5 (18.6 - 49.1)	100 (75.3 - 100)	73.8 (64.2 - 82.0)	37.0 (19.4 - 57.6)	88.9 (70.8 - 97.6)	58.5 (42.1 - 73.7)	76.9 (46.2 - 95.0)
Change B-lines > 2	97.4 (90.8 - 99.7)	67.5 (50.9 - 81.4)	93.1 (77.2 - 99.2)	85.1 (75.8 - 91.8)	25.9 (11.1 - 46.3)	88.9 (70.8 - 97.6)	54.5 (38.8 - 69.6)	70.0 (34.8 - 93.3)
Change B-lines > 3	94.7 (87.1 - 98.5)	87.5 (73.2 - 95.8)	89.7 (75.8 - 97.1)	93.5 (85.5 - 97.9)	22.2 (8.6 - 42.3)	100 (87.2 - 100)	56.2 (41.2 - 70.5)	100 (54.1 - 100)
Change B-lines < 2	0.0 (0.0 - 4.7)	67.5 (50.9 - 81.4)	26.2 (18.0 - 35.8)	0.0 (0.0 - 24.7)	63.0 (42.4 - 80.6)	11.1 (2.4 - 29.2)	23.1 (5.0 - 53.8)	41.5 (26.3 - 57.9)

BNP, B-type natriuretic peptide.

Supplementary Table 2. Additive diagnostic accuracy of exercise B-lines on top of different HFpEF scores and BNP in the diagnosis of HFpEF (Exercise stress echocardiography cohort)

Variable	C-index increase of peak B-lines on top of Variable + BNP	P-value	C-index increase of change B-lines on top of Variable + BNP	P-value
Variables				
H2FPEF score	0.136 (0.073 to 0.199)	<0.0001	0.122 (0.060 to 0.183)	0.0001
H2FPEF score ≥ 6	0.131 (0.069 to 0.192)	<0.0001	0.118 (0.060 to 0.176)	<0.0001
HFA-PEFF score (rest)	0.075 (0.025 to 0.126)	0.003	0.066 (0.020 to 0.113)	0.005
HFA-PEFF score (rest) ≥ 5	0.138 (0.074 to 0.202)	<0.0001	0.125 (0.065 to 0.185)	<0.0001
HFA-PEFF score (exercise)	0.061 (0.019 to 0.102)	0.004	0.051 (0.014 to 0.089)	0.008
HFA-PEFF score (exercise) ≥ 5	0.094 (0.043 to 0.146)	0.0003	0.081 (0.034 to 0.128)	0.0007

BNP, B-type natriuretic peptide.

Supplementary Table 3

Additive diagnostic accuracy of different exercise B-line cut-offs (B-line peak >5 and B-line change >3) on top of different HFpEF scores and BNP in the diagnosis of HFpEF (Exercise stress echocardiography cohort)

Variable	C-index increase of peak B-lines >5 on top of Variable + BNP	P-value	C-index increase of change B-lines >3 on top of Variable + BNP	P-value
Variables				
H2FPEF score	0.120 (0.056 to 0.184)	0.0003	0.090 (0.037 to 0.143)	0.0009
H2FPEF score ≥ 6	0.108 (0.052 to 0.165)	0.0002	0.084 (0.034 to 0.135)	0.001
HFA-PEFF score (rest)	0.057 (0.014 to 0.100)	0.010	0.042 (0.003 to 0.081)	0.034
HFA-PEFF score (rest) ≥ 5	0.115 (0.057 to 0.174)	0.0001	0.089 (0.037 to 0.141)	0.0008
HFA-PEFF score (exercise)	0.044 (0.010 to 0.078)	0.011	0.033 (0.001 to 0.065)	0.041
HFA-PEFF score (exercise) ≥ 5	0.073 (0.028 to 0.118)	0.002	0.057 (0.017 to 0.098)	0.005

Supplementary methods

Baseline clinical parameters

The following baseline characteristics were collected in both cohorts: age, sex, cardiovascular risk factors [hypertension, diabetes, dyslipidemia, obesity, family history of coronary artery disease (CAD), smoking habit, previous HF hospitalization, history of atrial fibrillation or atrial flutter. Additionally, information on baseline medications were collected (ACE-inhibitor, angiotensin receptor blockers, beta-blockers, statins, diuretics, aldosterone antagonists, nitrates). Signs and symptoms of HF were assessed by clinical examination. Measurements of arterial blood pressure (BP), heart rate (HR), weight, and body mass index, were also collected; a resting 12-lead electrocardiogram (ECG) was also performed.

Eligibility criteria

In the exercise stress echocardiography (ESE) cohort, participants with Heart failure (HF) with preserved ejection fraction (HFpEF) were haemodynamically stable; ESE was performed >8 weeks after any previous HF hospitalization. Given the limitations of natriuretic peptide diagnostic thresholds in diagnosing HFpEF(1), patients having BNP concentrations lower than the 35 pg/ml cut-off proposed by European HF guidelines(2) were included if they had had a previous HF hospitalization. Controls were hypertensive subjects (with other cardiovascular risk factors such as dyslipidaemia, diabetes, or smoking habit), free of history of HF or significant coronary or valvular heart disease. Inclusion criteria for both cases and controls were: (i) ability to perform bicycle ESE; (ii) sinus rhythm; (iii) good echocardiographic window; (iv) no pulmonary fibrosis, or other pulmonary diseases potentially hampering image acquisition (pleural effusion, severe emphysema, previous pneumectomy or lobectomy, pulmonary cancer or metastases).

The final subgroup of HFpEF patients (N=76) was extracted from a whole population which was made up of 92 HFpEF patients. Of these, 16 patients did not satisfy the inclusion criteria, 7 patients had atrial fibrillation, and 9 patients had severe pulmonary diseases, other conditions limiting image

acquisition, or poor echocardiographic window. In the control subgroup, all patients fulfilled the inclusion criteria. The study was approved by the local ethics committee and managed in accordance with Good Clinical Practice and the Declaration of Helsinki. All patients provided written informed consent.

In the cycle ergometer test (CET) cohort, both cases (age ≥ 65 years, already diagnosed with HFpEF following hospitalization for heart failure, LVEF $>50\%$) and controls (age ≥ 65 years, no history of HF, non-dyspneic) were scheduled with an indication for an exercise test. Exercise testing indication was not recorded. All controls underwent CET for risk stratification; for HFpEF patients, a CET was performed to assess exercise tolerance and to investigate potential factors contributing to worsening HF (ischemia, arrhythmia, BP profile). The design of E-LUS (Effort-Lung Ultra Sound, ClinicalTrials.gov Identifier NCT04644692) study envisaged the following exclusion criteria for both cases and controls: i) opposition to participate to the research project/the use of their data, ii) pulmonary or pleural pathology preventing reliable acquisition of lung ultrasound (LUS) images (e.g. pulmonary fibrosis, diffuse interstitial lung disease or lung cancer), iii) history of thoracic surgery, legal protection measures (guardianship, curator, judicial safeguard), iv) pregnancy, v) parturient women or nursing mothers, vi) inability to express consent, vii) psychiatric care, and viii) recent (i.e. <15 days) discharge from hospital for a worsening heart failure hospitalization. The final cohort of (N=54, 27 cases and 27 controls) was extracted from a whole population which was made up of 56 patients (28 cases and 28 controls). Of these, 2 patients satisfied the exclusion criteria. All patients provided written informed consent.

In the CET cohort, all HFpEF patients had a previous HF hospitalization; in the ESE cohort all patients HFpEF were diagnosed according to 2016 European HF guidelines(2), and the vast majority (90%) had a previous HF hospitalization.

Exercise stress echocardiography and bicycle stress test protocol

In the ESE cohort, after clinical examination, resting echocardiography and LUS were performed (see below); patients underwent submaximal ESE (supine, slightly tilted on their left side) on a tilting table using a cycle-ergometer, as proposed by Cardiff-MEDIA protocol (slightly modified to provide a suitable acquisition period to perform both echocardiography and LUS)(3). Submaximal ESE has a good feasibility, allowing image acquisition throughout exercise(4,5). Exercise started at an initial 15W workload, with 5 W increments every minute and maintaining a pedalling rate of 55–65 r.p.m. Once heart rate >100 b.p.m. was reached, workload was kept constant for ~5-6 min while echocardiography, and LUS imaging were performed; subsequently, a 10-minute recovery phase started. Echocardiography and lung ultrasonography were performed: 1) at rest; 2) during exercise, i.e. at the heart rate of >100 b.p.m, or whenever symptoms developed (whichever first); 3) during the last 5 min of recovery; ECG and BP were continuously monitored, and recorded every 2 minutes throughout the test. For echocardiographic images, at least three consecutive beats were acquired.

In the CET cohort, all patients underwent maximal exercise test according to the protocol recommended by current guidelines(6). Namely, a maximal graded cycling was performed starting at an initial workload of 25 W and lasting for 2 min; the workload increased stepwise by 25 W at 2-min intervals. The ECG and BP were continuously monitored. LUS scanning was performed in the upright position at rest (before exercise) and soon after exercise (within the first two minutes of the recovery phase).

In both exercise protocols, exercise testing was interrupted in the event of typical chest pain, constraining breathlessness, dizziness, muscular exhaustion, >10 mmHg drop in systolic BP or severe hypertension (systolic BP \geq 250 mmHg), development of significant ventricular arrhythmias, or ST depression.

Expanded echocardiographic methods

In the ESE cohort, all patients underwent detailed echocardiographic examination (Esaote-MyLab Alpha eHD Crystalline series 7400), and lung ultrasonography at rest and at the maximal workload sustained during exercise. LV structure and function were evaluated from standard apical four-chamber, two-chamber, and parasternal long- and short-axis views. M-mode linear measurements of LV dimensions and wall thicknesses were obtained in all patients at rest; LV mass was calculated by using the Cube formula(7). LV volumes, stroke volume (SV) and LVEF were calculated using the biplane method of disks (modified Simpson's rule)(8). All cardiac chamber volumes and mass measures were indexed to body surface area(8). Left atrial volume was assessed by the biplane area-length method from apical 2- and 4-chamber views at end-systole and was indexed to body surface area (LA volume index, LAVi).

Peak velocities of early (E) and late (A) waves of transmitral flow and the E-wave deceleration time (DT), were measured from the pulsed-wave Doppler obtained between the tips of the mitral leaflets. Septal and lateral mitral annular velocities (for measurement of e' and s') were obtained by pulsed tissue Doppler imaging (TDI). Average E/e' ratio was calculated using the peak E-wave velocity and the average of septal and lateral e'(9).

Right ventricular function was estimated by measuring tricuspid annular plane peak systolic excursion (TAPSE, in mm) using M-mode echocardiography, and lateral tricuspid annulus peak systolic velocity by TDI (s', cm/s). Estimation of PASP was performed using the Bernoulli formula according to tricuspid regurgitation velocity (TRV)(9), as suggested by current available recommendation diastolic stress echocardiography(4). Assessment of right atrial pressure via vena cava measurements will not be as it could be challenging during exercise. Three cardiac cycles were obtained from the standard apical long-axis view, 4- and 2-chamber views. In the ESE cohort, LUS examinations were performed with the method of the 28-scanning points, as previously described(10), immediately after echocardiogram. B-lines were defined as discrete, laser-like vertical hyperechoic reverberation artefacts arising from the pleural line, extending to the bottom of the screen without

fading, and moving synchronously with lung sliding (11). Usually, when a scanning site displays a few B-lines, they are easily discernible and easy to count. If B-lines were greater in number and confluent (i.e. not enough distinguishable to be counted), the percentage of the screen below the pleural line occupied by B-lines was considered, and then divided by 10. The sum of B-lines recorded in each of the 28 scanning points yields a score (ranging from 0 to 280) denoting the extent of extravascular fluid of the lung (12). As previously reported by our group(13), LUS reliability is excellent, with both intra- and inter-observer correlation coefficients ≥ 0.85 .

All echocardiographic images, performed using a 1–4 MHz phased-array probe, were recorded and analysed off-line. All exams were performed by a single operator (D.S.), blinded to patients' data and who did not take part in their clinical management. Typically, 3 minutes, or less, were necessary to perform echocardiographic acquisitions at peak exercise (focused on cardiac volumes, PASP, E/e' ratio) or LUS (overall, about 5-6 minutes for both techniques). The present study fulfilled all the applicable requirements reported in the Expert Consensus Document on LUS in HF studies (14).

In the CET cohort, echocardiographic examination was not planned by study design; LUS examinations were performed with the 8-zone protocol, as previously described(15), using a pocket-sized, hand-held ultrasound system (Vscan with phased-array transducer for the first half, and Vscan Extend with linear transducer for the second half of patients, GE Healthcare). All exams were performed by 4 physicians (2 cardiologists specialized in exercise testing and 2 residents in nuclear medicine), with limited or no experience with LUS ("LUS beginners"), unaware of group assignment, and who took no part in the clinical management. Two experienced cardiologists (N.G., M.E.) trained those operators on B-line assessment with a very short training (30 minutes of theory and practice on two patients). The 8-zone protocol, introduced in 2006 by Volpicelli, represents the most versatile scanning method, largely adopted in both acute and chronic settings (16,17). The 8-zone protocol consists of 2 anterior zones (from the sternum to the anterior axillary line, subdivided into upper and lower halves from the clavicle to the second-third intercostal spaces and from the third space to the diaphragm), and 2 lateral zones (from the anterior to the posterior axillary line, subdivided into upper

and basal halves) per side. Prior to the study, nursing staff had theoretical training by video as well as hands-on training led by experienced cardiologist (N.G., M.E.).

In both cohorts, scanning sites with missing B-line data (e.g. due to minimal pleural effusions or difficulty in detecting pleural sliding) were not taken into account and were consequently judged as 'zero B-lines'. The number of B-lines was assessed in real time; additionally, B-line clips were recorded in order to allow off-line analysis.

Calculation of H2FPEF and HFA-PEFF score

The H2FPEF score was calculated as previously described(18). Briefly, the H2FPEF score (ranging from 0 to 9) comprises the following categories: "Heavy" (obesity, BMI >30 Kg/m²), Hypertension (treatment with ≥ 2 antihypertensive), atrial Fibrillation (paroxysmal or persistent), Pulmonary artery systolic pressure >35 mm Hg, Elderly (age >60 years), and Filling (average E/e' ratio >9); each clinical variable score 1 point, except obesity (2 points), and atrial fibrillation (3 points). A score of 6 or more is able to diagnose HFpEF with 90% probability(18)

The HFA-PEFF score has previously been described in detail as part of the HFA-PEFF diagnostic algorithm for suspected HFpEF(19). The HFA-PEFF score includes the three following domains: (i) functional, (ii) morphological and (iii) biomarker. Each domain has major and minor criteria, individually scoring a maximum of 2 points. The highest HFA-PEFF score is 6, and an HFA-PEFF score of ≥ 5 is considered to be diagnostic of HFpEF. For the calculation of the HFA-PEFF score, we used resting parameters of laboratory and echocardiography data. In the ESE cohort, each echocardiographic or bio-humoral (i.e. BNP) parameter needed to assess HFA-PEFF score was available for all the patients (cases and controls). We also calculated an HFA-PEFF score incorporating echocardiographic indices of diastolic dysfunction from ESE as per Consensus Recommendations (2 points for exercise average E/e' ≥ 15 , 3 points for exercise average E/e' ≥ 15 and TRV >3.4 m/s)(19).

Statistical methods

The main analysis was performed in the ESE cohort, for which a complete echocardiographic, lung ultrasound, and laboratory dataset was available. In the nurse-led CET cohort, the analysis was focused on diagnostic value of lung ultrasound. Continuous variables are expressed as mean \pm standard deviation (SD), or median and interquartile range (IQR), as appropriate; categorical variables are presented as counts and percentages. Distribution of variables was visually checked. In both cohorts (i.e. ESE and CET cohorts), differences between HFpEF and controls in terms of demographic, clinical, echocardiographic, and lung ultrasound characteristics were assessed by unpaired t-test or Mann-Whitney test for continuous variables, and χ^2 test or Fisher's exact test for categorical variables, as appropriate.

In both cohorts, we calculated sensitivity, specificity, negative predictive value, positive predictive value of different cut-offs for exercise B-lines (peak and change B-lines); in the ESE cohort, we also tested relevant cut-offs for average E/e' (≥ 9 , ≥ 15), BNP (>35 pg/ml), H2FPEF score (≥ 6), and for rest and exercise HFA-PEFF (≥ 5)(19).

In the ESE cohort, receiver operating characteristic (ROC) curves were created and areas under the curves (AUC) were calculated in order to assess the ability of exercise B-lines and the three considered scores in diagnosing HFpEF. With regard to B-lines (peak and change), we also assessed the optimal cut-offs by Youden's index; we then tested those cut-offs in the CET cohort.

Calculation of C-index was used to assess the individual performance in diagnosing HFpEF for the following variables: average E/e' (rest, and exercise), BNP, H2FPEF score, HFA-PEFF scores (i.e. rest, and exercise), and B-lines (rest, peak and change B-lines). In the CET cohort, taking into account a possible learning curve effect, we also assessed C-index for exercise B-lines in the first half and the second half of consecutive patients. In addition, the increase in C-index was calculated to assess the additional diagnostic value of each variable (i.e. B-lines and other echocardiographic/bio-humoral variables) on top of the three score for HFpEF diagnosis. We also tested the increase in C-index of B-lines on top different models, including one of the three considered score and BNP. C-index was

overall assessed for all covariates, both as continuous and dichotomous (optimal and relevant cut-offs) variables. A P-value <0.05 was considered significant. Statistical analyses were performed using SPSS package version 25.0 (Chicago, L, USA) and R version 3.6.1 (the R foundation for Statistical Computing).

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