VIA MEDICA

ORIGINAL ARTICLE

Cardiology Journal
2024, Vol. 31, No. 2, 285–299
DOI: 10.5603/cj.92813

ISSN 1897–5593
eISSN 1898–018X

Application of homocysteine as a non-invasive and effort-free measurements for risk assessment of patients with pulmonary hypertension

Mei-Tzu Wang[1–3], Pei-Ling Chi[4], Chin-Chang Cheng[2, 5], Wei-Chun Huang[2, 3, 5]*, Lee-Wei Chen[1, 6, 7]*

[1]Institute of Emergency and Critical Care Medicine, National Yang Ming Chiao Tung University, Taipei City, Taiwan
[2]Department of Critical Care Medicine, Kaohsiung Veterans General Hospital, Kaohsiung City, Taiwan
[3]Department of Medicine, School of Medicine, National Yang Ming Chiao Tung University, Taipei City, Taiwan
[4]Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung City, Taiwan
[5]Department of Physical Therapy, Fooyin University, Kaohsiung, Taiwan
[6]Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan
[7]Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan

Abstract

Background: *Current guideline-recommended multiparameters used to assess the risk levels of pulmonary arterial hypertension (PAH) are invasive hemodynamic measurements or effort-dependent exercise tests. Serum natriuretic peptide is only one kind of effort-free biomarker that has been adopted for risk assessment. This study aimed to investigate the application of homocysteine as a non-invasive and effort-free measurement for the risk assessment of patients with PAH.*

Methods: *Samples of 50 patients diagnosed with PAH via right heart catheterization were obtained, and the patients were divided into low-, intermediate- and high-risk groups for further analysis. Additionally, serum N-terminal prohormone of B-type natriuretic peptide (NT-proBNP) and homocysteine levels of monocrotaline (MCT)-induced PAH rats were analyzed at each week with progressed severity of PAH, and they were sacrificed on day 28 with pathology being assessed.*

Results: *Hyperhomocysteinemia was an independent predictor (odds ratio [OR]: 1.256; 95% confidence interval [CI]: 1.002–1.574) and showed a linear correlation with NT-proBNP. Hyperhomocysteinemia could discriminate between low/intermediate and high-risk levels in PAH with a cut-off value in 12 μmol/L. Moreover, the elevated homocysteine levels by weeks in MCT rats also demonstrated the association between homocysteine and the severity of PAH.*

Conclusions: *Homocysteine can be a non-invasive and effort-free risk assessment for patients with pulmonary hypertension. Homocysteine level had a linear correlation with NT-proBNP level, and patients with hyperhomocysteinemia had a higher risk level, higher NT-proBNP level, and decreased lower diffusing capacity for carbon monoxide. The correlation between homocysteine level and PAH severity was also demonstrated in MCT rats.* (Cardiol J 2024; 31, 2: 285–299)

Keywords: biomarker, homocysteine, pulmonary hypertension, risk assessment

Address for correspondence: Wei-Chun Huang, MD, PhD, Department of Critical Care Medicine, Kaohsiung Veterans General Hospital, No. 386, Dazhong 1st Rd., Zuoying Dist., Kaohsiung City 813, Taiwan, tel: 886-7-3468278, fax: 886-7-3455045, e-mail: wchuanglulu@gmail.com; Lee-Wei Chen, MD, PhD, Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung, No. 386, Dazhong 1st Rd., Zuoying Dist., Kaohsiung City 813, Taiwan, tel: 886-7-3468278, fax: 886-7-3455045, e-mail: lwchen@vghks.gov.tw

Received: 19.11.2022 Accepted: 9.08.2023 Early publication date: 12.09.2023

**These authors contributed equally to this work.*

Introduction

Pulmonary arterial hypertension (PAH) is defined as mean pulmonary arterial pressure (mPAP) > 20 mmHg at rest as assessed by right heart catheterization, pulmonary arterial wedge pressure ≤ 15 mmHg, and pulmonary vascular resistance > 2 wood units, according to the classification of 2022 European Society of Cardiology (ESC)/European Respiratory Society (ERS) guidelines [1]. Pathologic progressions of vascular remodeling leads to pulmonary hypertension, right-sided heart failure, and death, once compensatory mechanisms have failed [2–4].

Most of the ESC/ERS recommended multiparameters for risk assessment and outcome prediction are invasive hemodynamic measurements or effort-dependent exercise tests except serum natriuretic peptide, B-type natriuretic peptide (BNP), or N-terminal prohormone of B-type natriuretic peptide (NT-proBNP), which is only one type of effort-free biomarker that has been adopted for risk assessment [5]. However, any kind of effort-dependent exercise tests are limited by a patient's physical restriction or exercise disability, an alternative or additional biomarker could provide more information of outcomes if invasive or effort-dependent tests are not accessible or available.

There are many circulating biomarkers involved in functional pathways associated with the pathobiology of pulmonary hypertension. Homocysteine is one of these circulating biomarkers, which involved several pathological functional pathways of PAH, and was considered to be correlated with diagnosis and prognosis of PAH [6, 7]. Elevated homocysteine levels have been toxic to the vascular endothelium and is an attribute for coronary disease, cerebrovascular disease, and peripheral vascular disease [8, 9]. However, the relationship between homocysteine and pulmonary hypertension remains unclear. This study aimed to investigate the application of homocysteine as an alternative or additional non-invasive and effort-free measurement in addition to serum natriuretic peptide for risk assessment of patients with pulmonary hypertension.

Methods

Samples in this study were obtained from the Kaohsiung Veterans General Hospital Biobank with approval from the respective ethics committees of Kaohsiung Veterans General Hospital. Deidentified data of patients diagnosed with pulmonary hypertension were analyzed to establish the association between circulating biomarkers and the risk levels of pulmonary hypertension. Pulmonary hypertension was defined as a mPAP ≥ 20 mmHg at rest, as assessed by right heart catheterization according to 2022 ESC/ERS guidelines [1].

Animal model

A monocrotaline (MCT)-induced PAH rat model was used in this study, and the Institutional Animal Care and Use Committee of Kaohsiung Veterans General Hospital approved the experimental protocols. Six-week-old male Sprague-Dawley rats in 220–280 g, were purchased from BioLASCO (Ilan, Taiwan) and handled according to the IACUC guidelines. To establish the MCT-induced PAH model, Sprague-Dawley rats were injected intraperitoneally with 60 mg/kg MCT (Sigma-Aldrich, St. Louis, MO, USA) as previously described [10, 11]. At the 1st, 2nd, 3rd, and 4th weeks, rat venous blood was drawn for analysis. On day 28, the animals were sacrificed, and PAH pathology was assessed as described previously [12]. All experimental protocols were performed in accordance with the European ethical regulation (Directive 2010/63/EU) and approved by the Institutional Animal Care and Use Committee, Kaohsiung Veterans General Hospital, Taiwan (Ref. 2019-2021-A054).

Serum NT-proBNP and homocysteine level of MCT rats

Rats were treated with phosphate-buffered saline or MCT (60 mg/kg) for 7, 14, 21, and 28 days. Blood samples were collected from the tail vein of the rats. Serum NT-proBNP and homocysteine concentrations were measured using ELISA kits (MBS2881463, MyBioSource, Inc., San Diego, CA, USA for NT-proBNP; MBS703069, MyBioSource, Inc., San Diego, CA, USA for homocysteine) according to the manufacturer's instructions.

Information about hemodynamic measurements of MCT rats, histology and immunohistochemical analysis of pulmonary arteries, blood tests assay for human, multiplex immunoassay of human blood, hemodynamics and cardiopulmonary function tests of human, and risk level assessment are presented in the **Supplementary Appendix**.

Ethics statement

The Institutional Review Board (IRB) of Kaohsiung Veterans General Hospital approved this study (No. KSVGH21-CT9-04). Written informed

consent was not required for this study as the Biobank research database consisted of de-identified secondary data for research purposes. The IRB of Kaohsiung Veterans General Hospital issued a formal written waiver of the requirement for informed consent.

Statistical analyses

SPSS version 22 (IBM Corp., Armonk, NY, USA) was used for data analysis. Percentile values were used to express categorical data and were analyzed using the chi-square test. Mean (μ) and standard deviation (SD) values were used for continuous variables using the Student unpaired test. Multiplex immunoassay biomarkers were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction, and statistical significance was defined as $p < 0.05$ after verifying the equality of variances.

Univariate and multivariate forward stepwise logistic regression analyses were performed to assess predictors for the high-risk group, and the odds ratios (OR) and the associated 95% confidence intervals (CI) for significant variables were calculated, and statistically significant predictor was set at $p < 0.05$. Correlation analysis was performed to assess the correlation between the biomarkers and NT-proBNP levels. To compare NT-proBNP and homocysteine levels with increasing severity of pulmonary hypertension by weeks following MCT infusion, ANOVA with post-hoc Fisher's least significant difference test was adopted after verifying the equality of variances. In addition, statistical significance was set at $p < 0.05$.

To find the most appropriate cut-off value for selective biomarker to determine the risk level for pulmonary hypertension, a receiver operating characteristic (ROC) analysis was performed. Moreover, different biomarkers combinations and the comparison between respective predictive value of each model were illustrated. The areas under the curves (AUC) were calculated.

Results

The basic characteristics of patients with pulmonary hypertension based on the ESC/ERS guideline-recommended risk assessment are reported in Table 1 [13]. There were 3 patients in low-risk group, 24 intermediate-risk patient, and 23 patients in high-risk group. There were no disparities in sex and age between the low/intermediate-risk and high-risk groups. Biochemistry panel demonstrated worse renal function blood urea nitrogen = 15.0 ± 4.5 vs. 24.0 ± ± 13.7 mg/dL, p = 0.006; serum creatinine = 0.9 ± ± 0.2 vs. 1.2 ± 0.5 mg/dL, p = 0.030) in high-risk group. With regard to circulating biomarkers, higher homocysteine (10.6 ± 4.0 vs. 17.0 ± 7.0 μmol/L, p = 0.005, Fig. 1A), uric acid (UA; 6.0 ± ± 1.7 vs. 7.7 ± 2.5 mg/dL, p = 0.006, Fig. 1B), D-dimer (744.8 ± 579.1 vs. 1,525.5 ± 1,559.7 ng/mL, p = 0.040, Fig. 1C), and C-reactive protein (CRP; 0.7 ± 0.7 vs. 2.6 ± 2.7 mg/dL, p = 0.007, Fig. 1D) were observed in the high-risk group. Despite no significant differences of multiplex immunoassay circulating biomarkers, including angiopoietin-2, bone morphogenetic protein (BMP)-2, BMP-4, cluster of differentiation 40 (CD40), endoglin, interlukin-6, myeloperoxidase, osteopontin, and vascular endothelial growth factor (VEGF), there was an increased trend by disease severities. Furthermore, Bonferroni correction was applied for analysis of multiplex immunoassay biomarkers (**Suppl. Table S1**), and the insignificance could be attributed to the small sample size.

Hemodynamics and cardiopulmonary function tests for pulmonary hypertension risk assessment based on the ESC/ERS guidelines are also listed in Table 1. Compared to reports in low/intermediate-risk group, the high-risk group was reported to have worse World Health Organization (WHO) functional (Fc III = 11.1% vs. 73.9%, p < 0.001), worse exercise and cardiopulmonary exercise capacity (six-minute walking distance [6MWD] = = 367.7 ± 102.6 vs. 251.4 ± 143.0 m, p < 0.001; VE/VCO_2 = 32.8 ± 7.4 vs. 41.3 ± 14.9, p = 0.049), higher NT-proBNP value (NT-proBNP = 794.5 ± ± 918.5 vs. 4390.6 ± 4843.6 pg/mL, p = 0.002). Regarding hemodynamic parameters, patients in the high-risk group had worse cardiac function (cardiac output = 5.4 ± 0.6 vs. 4.1 ± 1.5 L/min, p = 0.028; cardiac index = 3.6 ± 0.5 vs. 2.4 ± 0.9 L/min/m^2, p = 0.001), worse vascular saturation (pulmonary artery saturation = 73.3 ± 4.8 vs. 50.7 ± 15.1%, p = 0.007; superior vena cava saturation = 71.3 ± ± 6.6 vs. 57.5 ± 12.0%, p < 0.001; inferior vena cava saturation = 74.5 ± 8.7 vs. 54.9 ± 14.1%, p = = 0.005), and higher pulmonary vascular resistance (6.0 ± 3.4 vs. 10.9 ± 8.6 woods, p = = 0.034) compared to the reports of patients in the low/intermediate-risk group. With regard to pulmonary function tests, forced expiratory volume in the first second (FEV_1) and FVC (FEV_1 = 1.9 ± 0.8 vs. 1.4 ± 0.5% predicted, p = = 0.024; FVC = 2.3 ± 1.2 vs. 1.7 ± 0.6 L, p = 0.020) were lower in the high-risk group than in the low/intermediate-risk group.

Table 1. Basic characteristics, hemodynamics and cardiopulmonary function tests of patients with pulmonary hypertension based on risk levels.

Variables	Low/intermediate risk (n = 27)	High risk (n = 23)	P
Female	22.0 (81.5%)	20.0 (87.0%)	0.711
Age [years]	56.4 ± 14.7	63.7 ± 15.8	0.141
Body weight [kg]	60.4 ± 12.5	68.6 ± 25.0	0.166
Body height [cm]	156.5 ± 8.6	151.0 ± 22.5	0.279
Body surface area [m^2]	1.6 ± 0.2	1.7 ± 0.2	0.521
Hematology tests:			
White blood cells [K/μL]	6.8 ± 2.2	6.8 ± 2.2	0.992
Red blood cells [M/μL]	4.5 ± 0.5	4.6 ± 0.9	0.630
Hemoglobin [g/dL]	13.3 ± 1.7	13.3 ± 1.8	0.952
Hematocrit [%]	40.1 ± 4.3	41.1 ± 6.0	0.510
Red blood cell volume distribution [%]	14.8 ± 4.8	15.9 ± 4.3	0.424
Platelet [K/μL]	250.5 ± 96.2	204.8 ± 73.2	0.069
Neutrophil [%]	62.1 ± 13.6	65.4 ± 10.9	0.349
Lymphocyte [%]	28.2 ± 11.9	23.0 ± 10.1	0.108
Neutrophil/Lymphocyte ratio	3.2 ± 3.1	3.6 ± 2.2	0.625
Prothrombin time [s]	11.1 ± 1.3	18.1 ± 22.8	0.158
International normalized ratio	1.0 ± 0.1	1.2 ± 0.6	0.120
Partial thromboplastin time [s]	31.0 ± 4.6	30.5 ± 6.9	0.779
Biochemistry panel:			
Sodium [mmol/L]	141.2 ± 3.1	139.2 ± 3.9	0.043
Blood urea nitrogen [mg/dL]	15.0 ± 4.5	24.0 ± 13.7	0.006
Serum creatinine [mg/dL]	0.9 ± 0.2	1.2 ± 0.5	0.030
Estimated GFR [mL/min/1.73 m^2]	73.6 ± 14.3	62.2 ± 26.1	0.070
Fasting plasma glucose level [mg/dL]	100.2 ± 13.9	98.1 ± 32.7	0.796
Aspartate aminotransferase [U/L	30.7 ± 20.4	27.8 ± 12.3	0.542
Alanine aminotransferase [U/L]	25.9 ± 15.2	25.2 ± 18.2	0.874
Alkaline phosphatase [U/L]	62.0 ± 32.4	79.3 ± 32.6	0.105
Total bilirubin [mg/dL]	0.7 ± 0.5	0.9 ± 0.7	0.234
Albumin [g/dL]	4.1 ± 0.6	3.8 ± 0.6	0.070
Lactate dehydrogenase [U/L]	214.7 ± 89.2	212.6 ± 34.2	0.925
Lipid profile:			
Total cholesterol [mg/dL]	178.2 ± 40.9	162.3 ± 32.1	0.146
High-density lipoprotein [mg/dL]	50.5 ± 17.5	46.5 ± 16.2	0.413
Low-density lipoprotein [mg/dL]	96.0 ± 25.7	98.6 ± 30.6	0.751
Triglyceride [mg/dL]	120.3 ± 65.7	96.4 ± 37.3	0.154
Multiplex immunoassay circulating biomarkers:			
Angiopoietin-2 [pg/mL]	6237.3 ± 4790.3	5871.0 ± 5029.6	0.793
BMP-2 [pg/mL]	14.6 ± 0.0	12.4 ± 2.1	0.074
BMP-4 [pg/mL]	4.5 ± 0.6	5.1 ± 1.2	0.084
CD40 [pg/mL]	1689.6 ± 923.9	1666.3 ± 817.7	0.926
Endoglin [pg/mL]	1319.1 ± 541.6	1411.1 ± 433.6	0.522
Interlukin-6 [pg/mL]	2.5 ± 1.6	3.6 ± 6.5	0.452
Myeloperoxidase [pg/mL]	6476.3 ± 1897.4	6345.4 ± 1753.7	0.802
Osteopontin [pg/mL]	28901.9 ± 13600.6	35805.0 ± 34042.2	0.369
VEGF [pg/mL]	34.7 ± 23.8	26.2 ± 13.8	0.141
von Willebrand factor [%]	161.9 ± 53.5	164.8 ± 60.5	0.890

→

Table 1 (cont.). Basic characteristics, hemodynamics and cardiopulmonary function tests of patients with pulmonary hypertension based on risk levels.

	Low/intermediate risk (n = 27)	High risk (n = 23)	P
World Health Organization functional class III	3.0 (11.1%)	17.0 (73.9%)	< 0.001
Six-minute walking distance [m]	367.7 ± 102.6	251.4 ± 143.0	< 0.001
Cardiopulmonary exercise testing:			
Peak oxygen consumption [mL/min/kg]	74.4 ± 28.0	65.6 ± 25.4	0.315
VE/VCO_2	32.8 ± 7.4	41.3 ± 14.9	0.049
NT-proBNP [pg/mL]	794.5 ± 918.5	4390.6 ± 4843.6	0.002
Hemodynamics:			
Heart rate [bpm]	83.5 ± 16.5	85.9 ± 13.0	0.583
Right atrial pressure [mmHg]	11.7 ± 3.8	13.9 ± 6.3	0.235
Cardiac output [L/min, Thermodilution method]	5.4 ± 0.6	4.1 ± 1.5	0.028
Cardiac index [$L/min/m^2$, Thermodilution method]	3.6 ± 0.5	2.4 ± 0.9	0.001
Cardiac output [L/min, Fick formula]	4.3 ± 1.3	3.5 ± 1.4	0.162
Cardiac index [$L/min/m^2$, Fick formula]	2.7 ± 0.8	2.1 ± 0.8	0.082
Pulmonary artery saturation [%]	73.3 ± 4.8	50.7 ± 15.1	0.007
Superior vena cava saturation [%]	71.3 ± 6.6	57.5 ± 12.0	< 0.001
Inferior vena cava saturation [%]	74.5 ± 8.7	54.9 ± 14.1	0.005
Mean arterial pressure [mmHg]	97.7 ± 12.1	98.6 ± 12.3	0.802
Mean pulmonary arterial pressure [mmHg]	36.5 ± 14.1	43.6 ± 11.7	0.082
Pulmonary arterial wedge pressure [mmHg]	8.7 ± 6.5	11.0 ± 7.0	0.238
Pulmonary vascular resistance [woods]	6.0 ± 3.4	10.9 ± 8.6	0.034
Left ventricular ejection fraction [%]	59.8 ± 4.0	58.9 ± 2.7	0.365
Peak tricuspid regurgitation peak gradient [mmHg]	51.5 ± 16.0	59.6 ± 23.3	0.154
Pulmonary function tests:			
Total lung capacity [L]	4.6 ± 1.3	4.3 ± 0.9	0.587
FEV1 [s]	1.9 ± 0.8	1.4 ± 0.5	0.024
FEV1/FVC (% predicted)	82.0 ± 7.6	83.4 ± 13.0	0.667
Diffusing capacity for carbon monoxide (% predicted)	58.3 ± 25.8	50.8 ± 25.3	0.385

Data of continuous variables were expressed as mean ± standard deviation. Changes of categorical variables were analyzed by chi-square tests and were expressed by (N, %); BMP — bone morphogenetic protein; CD40 — cluster of differentiation 40; FEV1 — forced expiratory volume in first second; GFR — glomerular filtration rate; NT-proBNP — N-terminal prohormone of B-type natriuretic peptide; VEGF — vascular endothelial growth factor; VE/VCO_2 — ventilatory equivalents for carbon dioxide

Univariate (Table 2) and multivariate (Table 3) logistic regression analyses were performed to assess the predictors in the high-risk group. Multivariate logistic regression analysis demonstrated that homocysteine (OR: 1.256; 95% CI: 1.002––1.574, Table 3) was an independent predictor of high-risk levels. Furthermore, correlation analysis was performed to assess potential biomarkers that correlate with NT-proBNP levels (Table 4). Homocysteine ($\beta = 0.75$, $p < 0.001$) and UA ($\beta = 0.44$, $p = 0.002$) levels showed a good linear correlation with NT-proBNP levels. The linear correlation between NT-proBNP/homocysteine (Fig. 1E) and NT-proBNP/UA (Fig. 1F) was shown in Figure 1.

To find the most appropriate cut-off value for homocysteine for determining the risk level for pulmonary hypertension, a ROC analysis was performed. The best cut-off value was homocysteine = 12 μmol/L, the area under the ROC curve was 0.82, with a 95% CI between 0.67 to 0.97. Hyperhomocysteinemia (homocysteine > 12 μmol/L) could discriminate high-risk levels from low/intermediate-risk levels in pulmonary hypertension, with more high-risk patients (≤ 12: 18.8%; > 12: 70.6%, p = 0.003, Fig. 1G) in patients with hyperhomocysteinemia. Patients with homocysteine > 12 μmol/L also had higher NT-proBNP (803.0 ± 1,165.4 vs. 4,057.7 ± 5,230.9 pg/mL,

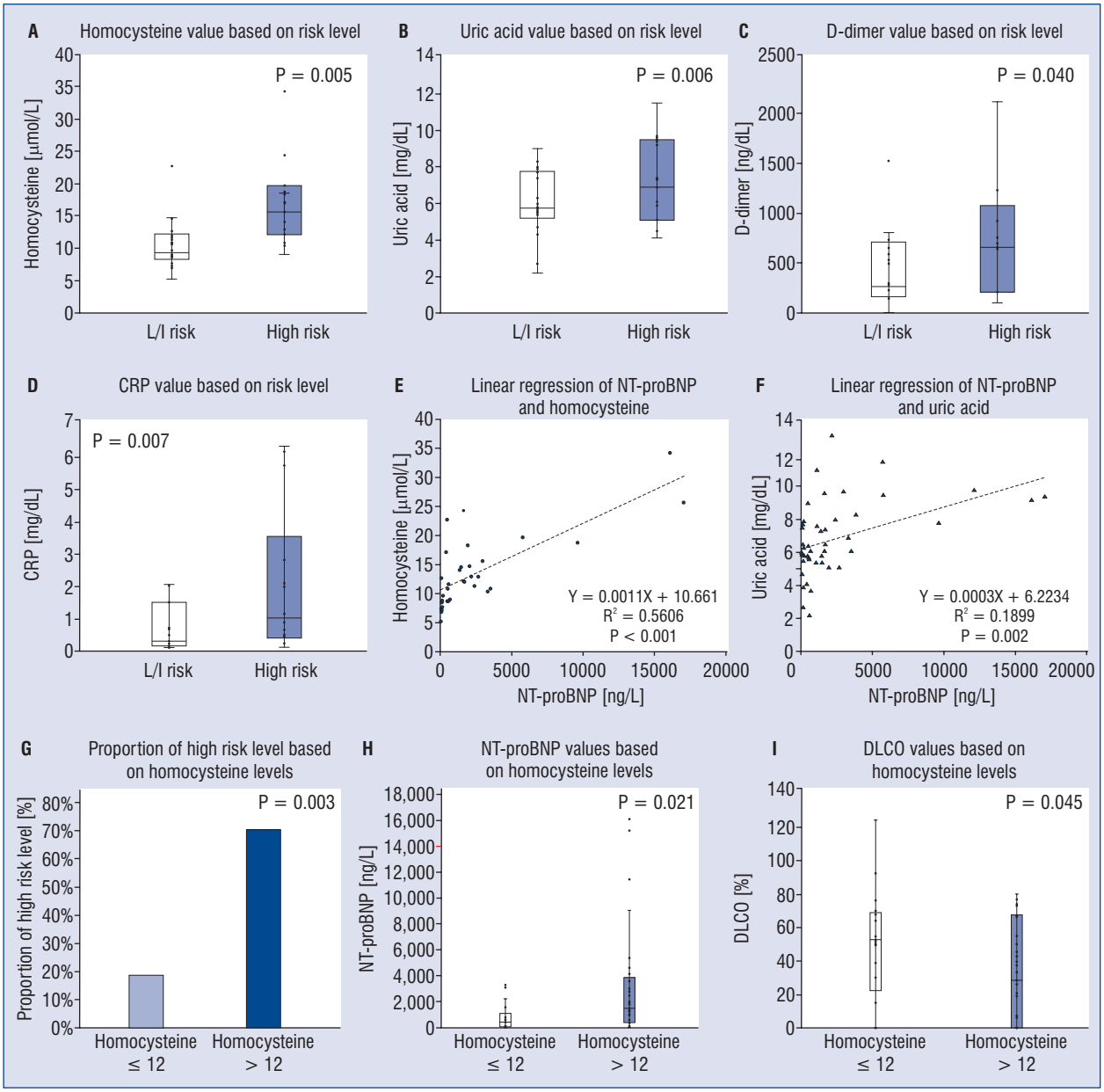

Figure 1. Circulating biomarkers of patients with pulmonary hypertension between low/intermediate (L/I) and high-risk groups and linear relationships between biomarkers and N-terminal prohormone of B-type natriuretic peptide (NT-proBNP) were illustrated in panels **A–F**. In the high-risk group, there were higher homocysteine (10.6 ± 4.0 vs. 17.0 ± ± 7.0 µmol/L, p = 0.005, **A**), higher uric acid (6.0 ± 1.7 vs. 7.7 ± 2.5 mg/dL, p = 0.006, **B**), higher D-dimer (744.8 ± ± 579.1 vs. 1,525.5 ± 1,559.7 ng/mL, p = 0.040, **C**), and higher C-reactive protein (CRP; 0.7 ± 0.7 vs. 2.6 ± 2.7 mg/dL, p = 0.007, **D**) levels than values in L/I-risk group; **E.** Linear relationship between NT-proBNP and homocysteine; **F.** Linear relationship between NT-proBNP and uric acid; **G–I.** Panels demonstrated that patients with pulmonary hypertension in hyperhomocysteinemia groups had a higher risk level. The best cut-off value acquired from receiver operating characteristic analysis was homocysteine = 12 µmol/L; **G.** Hyperhomocysteinemia (homocysteine > 12 µmol/L) could discriminate high-risk levels from L/I risk levels in pulmonary hypertension, with more high-risk patients (≤ 12:18.8%; > 12: 70.6%, p = 0.003) in patients with hyperhomocysteinemia; **H.** Patients with homocysteine > 12 µmol/L had higher NT-proBNP (803.0 ± 1,165.4 vs. 4,057.7 ± 5230.9 pg/mL, p = 0.021); **I.** Lower diffusing capacity for carbon monoxide (DLCO) (64.6 ± 24.6 vs. 44.2 ± 25.4% predicted, p = 0.045) was reported in patients with hyperhomocysteinemia.

Table 2. Univariate logistic regression analyses of predictive factors for high-risk level in pulmonary hypertension.

Variables	B	Standard error	Odds ratio	95% confidence interval	P value
Female	0.46	0.79	1.587	0.335–7.530	0.561
Age [years]	0.04	0.02	1.039	0.993–1.086	0.100
Body surface area [m^2]	0.91	1.37	2.477	0.170–36.118	0.507
Height [cm]	0.03	0.03	0.972	0.920–1.027	0.306
Weight [kg]	0.02	0.02	1.025	0.990–1.060	0.161
Heart rate [bpm]	0.01	0.02	1.009	0.971–1.048	0.659
Mean arterial pressure [mmHg]	0.00	0.02	1.002	0.956–1.051	0.919
Hematology tests:					
White blood cells [K/μL]	0.00	0.13	1.001	0.775–1.294	0.993
Red blood cells [M/μL]	0.15	0.40	1.156	0.532–2.512	0.714
Hemoglobin [g/dL]	0.01	0.17	1.005	0.728–1.388	0.976
Red blood cell volume distribution [%]	0.05	0.07	1.047	0.919–1.192	0.493
Platelet [K/μL]	0.01	0.00	0.993	0.986–1.001	0.071
Neutrophil [%]	0.03	0.03	1.035	0.983–1.089	0.192
Lymphocyte [%]	0.06	0.03	0.947	0.893–1.003	0.063
Neutrophil/Lymphocyte ratio	0.26	0.17	1.290	0.922–1.805	0.137
Prothrombin time [s]	0.32	0.19	1.370	0.941–1.995	0.100
Partial thromboplastin time [s]	0.01	0.05	0.986	0.893–1.088	0.775
Biochemistry panel:					
Na [mmol/L]	0.20	0.11	0.822	0.667–1.011	0.064
Estimated GFR [mL/min/1.73 m^2]	0.03	0.02	0.973	0.945–1.003	0.074
Aspartate aminotransferase [U/L]	0.01	0.02	0.989	0.955–1.024	0.541
Alanine aminotransferase [U/L]	0.00	0.02	0.996	0.963–1.031	0.835
Alkaline phosphatase [U/L]	0.02	0.01	1.019	0.995–1.043	0.127
Total bilirubin [mg/dL]	0.62	0.53	1.852	0.658–5.214	0.243
Albumin [g/dL]	0.94	0.54	0.392	0.135–1.138	0.085
Lactate dehydrogenase [U/L]	0.00	0.01	1.000	0.989–1.010	0.925
Lipid profile:					
High-density lipoprotein [mg/dL]	0.01	0.02	0.986	0.952–1.022	0.441
Low-density lipoprotein [mg/dL]	0.00	0.01	1.003	0.983–1.025	0.744
Total cholesterol [mg/dL]	0.01	0.01	0.988	0.971–1.005	0.162
Triglyceride [mg/dL]	0.01	0.01	0.991	0.979–1.004	0.190
Circulating biomarkers:					
Angiopoietin-2	0.00	0.00	1.000	1.000–1.000	0.765
BMP-2	6.15	6396.08	0.002	0.000–0.000	0.999
BMP-4	0.90	0.49	2.467	0.945–6.439	0.065
CD40	0.00	0.00	1.000	0.999–1.001	0.797
Endoglin	1.00	1.00	1.000	0.999–1.002	0.449
Interlukin-6	0.07	0.09	1.067	0.900–1.266	0.453
Myeloperoxidase	0.00	0.00	1.000	1.000–1.000	0.908
Osteopontin	0.00	0.00	1.000	1.000–1.000	0.360
VEGF	0.00	0.00	1.000	1.000–1.000	0.360
Homocysteine [μmol/L]	0.26	0.10	1.293	1.054–1.586	0.014
von Willebrand factor [%]	0.00	0.01	1.001	0.988–1.014	0.886
Uric acid [mg/dL]	0.41	0.17	1.509	1.088–2.094	0.014
D-dimer [ng/mL]	0.00	0.00	1.001	1.000–1.002	0.058

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Table 2 (cont.). Univariate logistic regression analyses of predictive factors for high-risk level in pulmonary hypertension.

Variables	B	Standard error	Odds ratio	95% confidence interval	P value
LVEF [%]	–0.07	0.09	0.935	0.790–1.106	0.431
Peak tricuspid regurgitation peak gradient [mmHg]	0.02	0.02	1.022	0.992–1.053	0.152
Pulmonary function tests:					
Total lung capacity [L]	–0.24	0.42	0.787	0.348–1.783	0.566
FEV1	–1.12	0.50	0.328	0.122–0.880	0.027
FEV1/FVC (% predicted)	0.01	0.03	1.013	0.957–1.073	0.654
Diffusing capacity for carbon monoxide (% predicted)	–0.02	0.02	0.981	0.953–1.011	0.218

BMP — bone morphogenetic protein; CD40 — cluster of differentiation 40; FEV1 — forced expiratory volume in first second; GFR — glomerular filtration rate; LVEF — left ventricular ejection fraction; VEGF — vascular endothelial growth factor

Table 3. Multivariate logistic regression analyses of predictive factors for high-risk level in pulmonary hypertension.

Variables	B	SE	OR	95% CI	P value
Homocysteine [μmol/L]	0.20	0.10	1.256	1.002–1.574	0.048
Uric acid [mg/dL]	0.30	0.20	1.338	0.834–2.147	0.227
FEV1 (L)	–1.00	0.60	0.378	0.120–1.193	0.097

CI — confidence interval; FEV1 — forced expiratory volume in first second; OR — odds ratio; SE — standard error

Table 4. The correlation between N-terminal prohormone of brain natriuretic peptide and circulating biomarkers.

Variables	Unstandardized coefficient			P value
	B	Standard error	β	
Angiopoietin-2 [pg/mL]	–0.04	0.11	–0.05	0.759
BMP-2 [pg/mL]	–287.03	442.25	–0.28	0.545
BMP-4 [pg/mL]	921.35	591.51	0.26	0.129
CD40 [pg/mL]	0.18	0.65	0.04	0.783
Endoglin [pg/mL]	0.02	1.17	0.00	0.988
Interlukin-6 [pg/mL]	–51.42	120.84	–0.06	0.672
Myeloperoxidase [pg/mL]	–0.04	0.35	–0.02	0.905
Osteopontin [pg/mL]	0.00	0.02	0.00	0.978
VEGF [pg/mL]	–18.76	36.81	–0.07	0.613
Homocysteine [μmol/L]	489.53	77.85	0.75	< 0.001
Uric acid [mg/dL]	750.24	233.61	0.44	0.002

BMP — bone morphogenetic protein; CD40 — cluster of differentiation 40; VEGF — vascular endothelial growth factor

p = 0.021, Fig. 1H) and lower diffusing capacity for carbon monoxide (DLCO) (64.6 ± 24.6 vs. 44.2 ± ± 25.4% predicted, p = 0.045, Fig. 1I).

The MCT-rat model was obtained successfully and reflected by the elevated right ventricular systolic pressure (21.4 ± 3.0 vs. 44.8 ± 9.0 mmHg, p = 0.001, Fig. 2B) and right ventricular hypertrophy (Fultons's index: 25.2 ± 2.8 vs. 49.1 ± 12.5%, p = 0.003, Fig. 2C) indicated by a significantly increased Fultons's index. MCT rats demonstrated

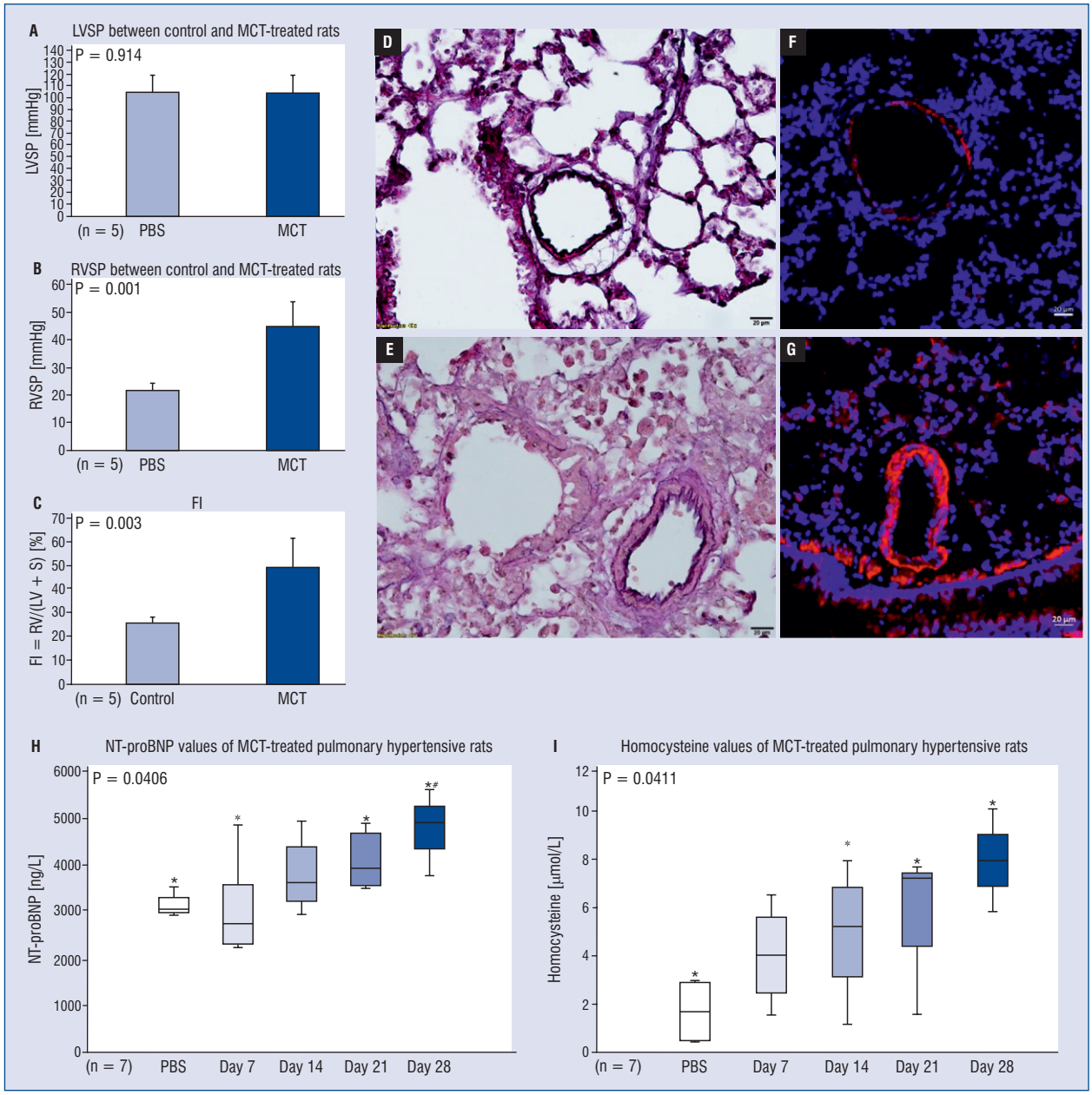

Figure 2. Hemodynamic measurements, histology, immunohistochemical analysis of pulmonary arteries in monocrotaline (MCT) rats; N-terminal prohormone of B-type natriuretic peptide (NT-proBNP) and homocysteine levels between control and MCT-induced pulmonary hypertensive rats. Rats were treated with MCT (60 mg/kg) for 28 days (n = 7 per group), and blood sampling was obtained from the tail vein of rats. Compared to rats treated with phosphate-buffered saline (PBS), rats treated with MCT (60 mg/kg) for 28 days (n = 5 per group) had similar left ventricular systolic pressure (LVSP) (**A**), but elevated right ventricular systolic pressure (RVSP) (**B**) and elevated Fulton's index (FI) with a higher ratio of right ventricular (RV) weight to left ventricular (LV) plus septal weight (RV/LV+S) (**C**). Elastica van Gieson staining revealed increased muscularization **(E)** and occluded pulmonary arteries in MCT-induced rats compared to the control group (**D**). Immunofluorescence staining of alpha-smooth muscle actin in lung sections from MCT-treated rats (**G**) demonstrated proliferated pulmonary arterial smooth muscle cells compared to PBS rats (**F**). NT-proBNP (**H**) and homocysteine (**I**) values were elevating with the severity of pulmonary hypertension by weeks. There were significant differences of NT-proBNP (p = 0.0406) and homocysteine (p = 0.0411) values with increasing severity of pulmonary hypertension by weeks following MCT infusion; *,#: ANOVA with post-hoc least significant difference test revealed a statistical difference between the marked groups.

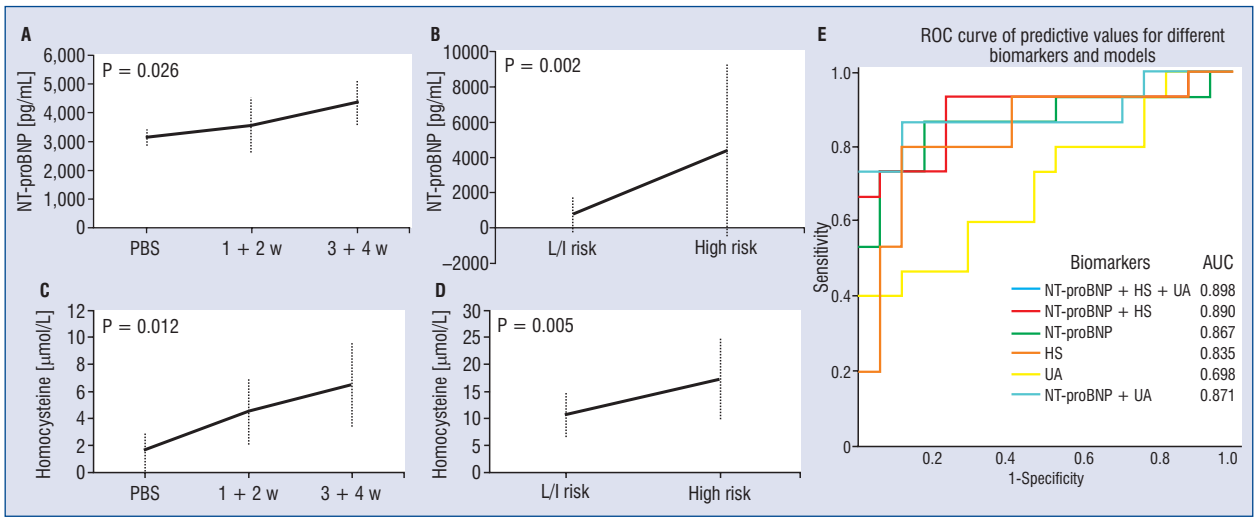

Figure 3. Comparative association of N-terminal prohormone of B-type natriuretic peptide (NT-proBNP) and homocysteine level between monocrotaline (MCT) pulmonary arterial hypertension (PAH) rats and PAH humans by disease severity. The PAH severity progressed by weeks for MCT rats; **A.** NT-proBNP level of MCT rats by weeks demonstrated higher NT-proBNP by progressed severity (p = 0.026); **B.** NT-proBNP level of PAH humans by severity. There was a higher NT-proBNP level in the high-risk group than in the low/intermediate (L/I) risk group (p = 0.002); **C.** Homocysteine level of MCT rats by weeks. Higher homocysteine level was demonstrated in 3 and 4 weeks MCT rats compared with the values in first 2 weeks (p = 0.012); **D.** Homocysteine level of PAH humans by severity. There was higher homocysteine value of high-risk patients than in the low/intermediate (L/I) group (p = 0.005); **E.** Different biomarker combinations and the comparison between respective predictive value of each model were illustrated. The areas under the curves (AUC) were calculated. NT-proBNP + homocysteine (HS) + uric acid (UA) had strongest predictive value (AUC = 0.898), following by NT-proBNP + HS (AUC = 0.890), NT-proBNP + UA (AUC = 0.871), NT-proBNP (AUC = 0.867), HS (AUC = 0.835), and then UA (AUC = 0.698); PBS — phosphate-buffered saline; w — weeks.

the elevation of NT-proBNP (Fig. 2H) and homocysteine (Fig. 2I) levels with progressed severity of pulmonary hypertension by weeks. Comparative association of NT-proBNP and homocysteine level between MCT rats and humans by disease severity were illustrated in Figure 3A–D. In addition, different biomarker combinations and the comparison between respective predictive value of each model were illustrated in Figure 3E. NT-proBNP + homocysteine + UA had strongest predictive value (AUC = 0.898), following by NT-proBNP + homocysteine (AUC = 0.890), NT-proBNP + UA (AUC = = 0.871), NT-proBNP (AUC = 0.867), homocysteine (AUC = = 0.835), and then UA (AUC = 0.698).

Discussion

This study aimed to identify potential biomarkers correlated and comparable to the current guidelines recommending NT-proBNP. A higher homocysteine level was an independent predictor for high-risk levels, and it showed a linear correlation with NT-proBNP. Further analysis indicated that the most appropriate cut-off value of homocysteine for risk level discrimination of pulmonary hypertension was homocysteine = 12 μmol/L.

The rationale for exploring biomarkers compatible and comparable with NT-proBNP

There was no single attribution of regulators or signaling molecules has adequate capacity to estimate the risk [6, 7, 14]. Currently, both the U.S. REVEAL risk score and the ESC/ERS guidelines are the most widely used multidimensional tools for risk assessment [13]. Among these, right heart catheterization is the only test to obtain the precise hemodynamic parameters for diagnosis and therapies [5].

Surprisingly, a previous study reported that BNP or NT-proBNP had a 98% sensitivity for excluding high right atrial pressure (≥ 8 mmHg) and low cardiac index (< 2.5 L/min/m^2), and in circumstances of extreme low BNP (< 50 pg/mL) or NT-proBNP (< 300 pg/mL) level, hemodynamic measurements no longer had independent prog-

nostic predictive values [14]. Moreover, COMPERA and the SPAHR registries demonstrated that the ability of mortality prediction is excellent even when only about a third of patients are followed up under the assessment of right heart catheterization [15, 18]. Nevertheless, due to the complexity of pulmonary hypertension, any single biomarker is insufficient for the broad assessment of patients with different etiologies of pulmonary hypertension. This study aimed to explore potential biomarkers compatible and comparable with NT-proBNP for disease follow-up.

The investigations of novel biomarkers and application of homocysteine for PAH risk assessment

The investigations of novel biomarkers, such as angiopoietin-2, BMP-2, BMP-4, CD40, endoglin, interlukin-6, myeloperoxidase, and osteopontin are currently in progress [7, 19, 20]. Angiopoietin-2 is produced by vascular smooth muscle cells and is involved in vascular damage/remodeling, and expression of angiopoietin-2 was up-regulated in plexiform lesions PAH lung tissues [21]. BMP-2 and BMP-4 exert opposing roles in the hypoxic pulmonary vasculature mediated by increasing endothelial nitric oxide synthase expression and activity, and BMP-2 has suggested protective effect [22, 23]. CD40 is a type I transmembrane receptor and one of the members of the tumor necrosis factor superfamily, which is expressed on epithelial cells, fibroblasts, endothelia cells, vascular smooth muscle cells, and platelets. The expression of CD40 promotes pro-thrombotic and pro-inflammatory effects, and is associated with systemic sclerosis and PAH [24, 25]. Endoglin and VEGF are angiogenic modulatory factors [26, 27]. Interlukin-6 is associated with vascular remodeling and development of PAH, which is able to predict poor adverse outcomes within the following year [28, 29]. Myeloperoxidase is able to reduce the bioavailability of nitric oxide, which is an important anti-inflammatory and vasodilating molecule. It also predicts outcomes in patients with PAH [30]. Osteopontin is involved in tissue remodeling, inflammation, and metastasis, which is recognized in cardiomyocytes and fibroblasts. Previous studies supported its correlation with mPAP, NT-proBNP, 6MWD and function class [31–33]. Bonferroni correction was applied for analysis of multiplex immunoassay biomarkers (**Suppl. Table S1**); despite having no significant statistical difference between low-, intermediate- and high-risk groups, the increased trend by disease severity was demonstrated. The insignificance could be attributed to the small sample size.

This study demonstrated that patients in high-risk group for pulmonary hypertension had higher homocysteine, UA, D-dimer, and CRP base on univariate analysis (Table 2). However, multivariate logistic regression analysis demonstrated that homocysteine (OR: 1.256; 95% CI: 1.002–1.574, Table 3) was the only independent predictor for high-risk levels. In addition, studies in animals and in cell cultures also demonstrated that homocysteine has a variety of toxic effects on the vasculature, endothelial dysfunction, medial remodeling and adventitial inflammation [34–41] which supports the result of serum homocysteine level of MCT rats in the present study.

In comparison with angiopoietin-2, BMP-2, BMP-4, CD40, endoglin, interlukin-6, myeloperoxidase, and osteopontin, which need to be acquired by multiplex immunoassay of human blood and were not feasible in clinical tests, homocysteine is available in daily clinical care. Furthermore, homocysteine impairs endothelium-dependent vasodilatation and is an endogenous inhibitor of nitric oxide synthase. Moreover, increased homocysteine level in PAH was also reported in a previous study [42–44]. In addition, comparison between each model illustrated in Figure 3E reported higher predictive value of homocysteine (AUC = 0.835) compared to uric acid (AUC = 0.698). Therefore, homocysteine rather than other biomarkers was selected for final advanced analysis under the consideration of multivariate analysis and clinical feasibility compared to other biomarkers.

Correlation between homocysteine and NT-proBNP, and application of homocysteine for follow-up of pulmonary hypertension

Homocysteine interferes with the expression of endothelial nitric oxide synthetase, with which its multifactorial attributions increase vascular thickness and activate elastin fragmentation, which eventually leads to PAH [8, 45]. Pulmonary hypertension can develop rapidly under hypoxic situations, and hyperhomocysteinemia was reported in cyanotic PAH patients compared to non-cyanotic patients [42–44]. A low DLCO could be seen in patients with primary pulmonary hypertension and other pulmonary vascular diseases with or without the restriction of lung volumes [46]. Moreover, lower DLCO (< 45%) was demonstrated in PAH patients with lower arterial oxygen tension [47]. Low DLCO was also an index of worse prognosis, a strong and independent risk factor for survival

in patients with pulmonary hypertension [48–50]. Hyperhomocysteinemia is an index for hypoxia and low DLCO [51]. This study reported that higher homocysteine group had more high-risk level patients (≤ 12 μmol/L: 18.8%; > 12 μmol/L: 70.6%, p = 0.003, Fig. 1G), and higher NT-proBNP (803.0 ± 1,165.4 vs. 4,057.7 ± 5,230.9 pg/mL, p = 0.021, Fig. 1H). This result supported the possibility of using homocysteine for disease follow-up.

A previous study demonstrated that higher homocysteine levels were correlated with higher concentrations of NT-proBNP when the differences were assessed in comparison with the upper quartile (≥ 18 μmol/L) with the lower quartile (≤ 12 μmol/L) [52]. Hyperhomocysteinemia predicted high NT-proBNP values via a link with impaired mitochondrial fatty oxidation [52]. Furthermore, homocysteine was one of the determinants of natriuretic peptide which was analyzed by univariate analyses [52]. Association between the log of plasma concentration of homocysteine and BNP was demonstrated with a correlation coefficient of +0.297 (95% CI: +0.097–+0.474, p = 0.004) [52]. In addition, homocysteine was reported to stimulate myocardial BNP and induce adverse left ventricular remodeling [53]. However, studies describing the correlation between homocysteine and NT-proBNP through a link with pulmonary hypertension are rare. This study showed that homocysteine had a linear correlation with NT-proBNP levels (β = 0.75, p < 0.001, Fig. 1E). The 1-year mortality was < 5%, 5–20%, and > 20% if NT-proBNP values are < 200, 300–1100, > 1100 pg/mL illustrated in 2022 ESC guideline [1]. As long as the biomarker identified had a good correlation with NT-proBNP, it was able to represent the estimated 1-year mortality as well as NT-proBNP does.

With regard to the use of homocysteine for pulmonary hypertension follow-up or severity evaluation, the current study demonstrated that hyperhomocysteinemia was present in pulmonary hypertension associated with the congenital heart disease group compared to the non-pulmonary hypertension group [42]. In addition, elevated total plasma homocysteine was reported in primary pulmonary hypertension patients compared to the control group (14.7 ± 7.2 vs. 10.2 ± 5.1, p = = 0.027), with the cut-off value of 15 μmol/L [54]. Hyperhomocysteinemia is a crucial factor in the pathogenesis of primary pulmonary hypertension as well as poor renal function [54]. These results support the present study, that a higher homocysteine value was reported in the high-risk group compared to the low/intermediate-risk group, and the most appropriate cut-off value based on ROC analysis was homocysteine = 12 μmol/L. In light of the small sample size of this study and ethical consideration, the present study used MCT induced PAH rats to evaluate the accessibility and reliability of using homocysteine to predict PAH risk level. The result proved a comparative association between disease severity and homocysteine level, which were demonstrated both in rats and humans (Fig. 3A–D).

Limitations of the study

Individual differences of metabolism and increased lipid profiles may interfere with homocysteine values. In addition, the acquisition of blood samples depends on the interval between application and permission. Blood samples of the present study were stored for an average of between 2 weeks and 1 month. The half-life of each biomarker and accuracy might affect the results of measurement. However, this was restricted by experimental accessibility and was a limitation of the present study. In addition, the small sample size of this study limits the reliability of the application of homocysteine as an index of risk assessment. Further investigation is needed to validate this study's result.

Study strength

Previous studies have illustrated the correlation between hyperhomocysteinemia and high NT-proBNP value via a link with impaired mitochondrial fatty oxidation. However, the correlation between homocysteine and NT-proBNP through a link with pulmonary hypertension has been obscured. Based on previous evidence, hyperhomocysteinemia were related with hypoxia-induced pulmonary vascular constriction and pulmonary hypertension. This study demonstrated that homocysteine had a linear correlation with NT-proBNP. These results posed a potential new circulating biomarker to achieve more accurate risk assessment of pulmonary hypertension.

Conclusions

This study demonstrated that patients with higher homocysteine levels had higher risk levels, higher NT-proBNP levels, and lower DLCO. This study also delineated a linear correlation between homocysteine and NT-proBNP levels. In summary, homocysteine can help discriminate between low/ /intermediate and high-risk groups. It is a potential

biomarker that could be compatible and comparable with NT-proBNP as a non-invasive and effort-free measurement for risk assessment and disease follow-up in pulmonary hypertension.

Acknowledgments

The authors appreciated the assistance of the Biobank, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, for processing of clinical specimens.

Funding

This study was supported by grants from the Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan, i.e., Grant Nos. KSVGH111-007, KSVGH110-077, VGHKS109-132, VAC110-001-4 and the Ministry of Science and Technology, i.e., grant numbers: MOST107-2314-B-075B-008-MY2 and MOST108-2314-B-075B-007-MY2.

Conflict of interest: None declared

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