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BASIC SCIENCES

Plasma Proteomics Identifies B2M as a Regulator of Pulmonary Hypertension in Heart Failure With Preserved Ejection Fraction

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BACKGROUND: Pulmonary hypertension (PH) represents an important phenotype in heart failure with preserved ejection fraction (HFpEF). However, management of PH-HFpEF is challenging because mechanisms involved in the regulation of PH-HFpEF remain unclear.

METHODS: We used a mass spectrometry–based comparative plasma proteomics approach as a sensitive and comprehensive hypothesis-generating discovery technique to profile proteins in patients with PH-HFpEF and control subjects. We then validated and investigated the role of one of the identified proteins using in vitro cell cultures, in vivo animal models, and independent cohort of human samples.

RESULTS: Plasma proteomics identified high protein abundance levels of B2M (β2-microglobulin) in patients with PH-HFpEF. Interestingly, both circulating and skeletal muscle levels of B2M were increased in mice with skeletal muscle SIRT3 (sirtuin-3) deficiency or high-fat diet–induced PH-HFpEF. Plasma and muscle biopsies from a validation cohort of PH-HFpEF patients were found to have increased B2M levels, which positively correlated with disease severity, especially pulmonary capillary wedge pressure and right atrial pressure at rest. Not only did the administration of exogenous B2M promote migration/proliferation in pulmonary arterial vascular endothelial cells but it also increased PCNA (proliferating cell nuclear antigen) expression and cell proliferation in pulmonary arterial vascular smooth muscle cells. Finally, *B2m* deletion improved glucose intolerance, reduced pulmonary vascular remodeling, lowered PH, and attenuated RV hypertrophy in mice with high-fat diet–induced PH-HFpEF.

CONCLUSIONS: Patients with PH-HFpEF display higher circulating and skeletal muscle expression levels of B2M, the magnitude of which correlates with disease severity. Our findings also reveal a previously unknown pathogenic role of B2M in the regulation of pulmonary vascular proliferative remodeling and PH-HFpEF. These data suggest that circulating and skeletal muscle B2M can be promising targets for the management of PH-HFpEF.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: beta 2-microglobulin ■ heart failure ■ hypertension, pulmonary ■ proteomics ■ sirtuin 3

Heart failure with preserved ejection fraction (HFpEF) is characterized by abnormal active relaxation and increased passive stiffness of the left ventricle (LV), resulting in elevated LV filling pressure. Passive backward transmission of the elevated LV filling pressure eventually leads to an increase in pulmonary (both venous and arterial) remodeling and pressures, which, over time, contribute to elevated pulmonary vascular resistance and right heart failure (HF).[1,2] Pulmonary hypertension (PH) is, therefore, a common complication in patients with HFpEF

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Nonstandard Abbreviations and Acronyms

B2M	β2-microglobulin
BNP	B-type natriuretic peptide
BTBD16	BTB/POZ domain-containing protein 16
CA3	carbonic anhydrase 3
CHGA	chromogranin-A
CRP	C-reactive protein
ET-1	endothelin-1
HF	heart failure
HFD	high-fat diet
HFE	hemochromatosis gene
HFpEF	heart failure with preserved ejection fraction
HIF-1α	hypoxia-inducible factor 1α
IFN-γ	interferon-γ
IL	interleukin
LV	left ventricle
MHC I	major histocompatibility complex class I
mPAP	mean pulmonary artery pressure
MS	mass spectrometry
NT-proBNP	N-terminal pro-B-type natriuretic peptide
Ob-Su	SU5416-exposed obese ZSF1
PCNA	proliferating cell nuclear antigen
PCWP	pulmonary capillary wedge pressure
PH	pulmonary hypertension
RAP	right atrial pressure
RHC	right heart catheterization
RVSP	right ventricular systolic pressure
SAA1	serum amyloid A1
SGLT2	sodium/glucose cotransporter 2
SIRT3	sirtuin-3
TMT	tandem mass tag
VEGF-D	vascular endothelial growth factor-D
WT	wild type

Highlights

- B2M (β2-microglobulin) is present at higher levels in patients with pulmonary hypertension–heart failure with preserved ejection fraction compared with control subjects free of heart failure, pulmonary hypertension, chronic kidney disease, cancers, and a few other rare diseases.
- Circulating and skeletal muscle expression levels of B2M correlate with pulmonary hypertension–heart failure with preserved ejection fraction severity, especially pulmonary capillary wedge pressure and right atrial pressure at rest.
- Loss of B2M improves glucose intolerance, attenuates pulmonary vascular proliferative remodeling, reduces pulmonary hypertension, and decreases right ventricular hypertrophy in mice with metabolic syndrome–associated pulmonary hypertension–heart failure with preserved ejection fraction.

(PH-HFpEF, group 2 PH) with a wide range of prevalence from 23% to 83%, depending on the definitions used.[2] SGLT2 (sodium/glucose cotransporter 2) inhibitors have recently been approved for the treatment of HFpEF.[3] Although SGLT2 inhibitors have been reported to improve PH in Ob-Su (SU5416-exposed obese ZSF1) rats, patients with type 2 diabetes, or HF,[4–6] the search for effective therapies for PH-HFpEF remains. A deeper insight into pathophysiological processes and molecular mechanisms may reveal new candidate targets for early identification and effective management of PH-HFpEF.

Apart from the gold standard right heart catheterization (RHC) and echocardiographic data, BNP (B-type natriuretic peptide) and NT-proBNP (N-terminal pro-B-type natriuretic peptide) are at present the only guideline-recommended biomarkers for the diagnosis and risk stratification of HF and PH.[7–9] High circulating levels of BNP and NT-proBNP appear to correlate well with increased frequency of HF hospitalizations, impaired RV function, and severe exercise intolerance in patients with PH-HFpEF.[10–12] However, disproportionately low BNP and NT-proBNP levels have been reported among patients with obesity, which coexists in up to 50% of patients with PH-HFpEF, complicating the usefulness of these factors in identifying at-risk subjects and predicting patient outcomes.[13] Elevated plasma levels of IL (interleukin)-1β, ET-1 (endothelin-1), VEGF-D (vascular endothelial growth factor-D), leptin, and adiponectin, as well as reduced serum levels of microRNA-206, have been associated with PH severity in humans and in animal models of PH-HFpEF.[14–20] However, their usefulness in the characterization and determination of disease management of PH-HFpEF remains unclear.

Here, we used a mass spectrometry (MS)–based comparative bottom-up plasma proteomics approach as a sensitive and comprehensive hypothesis-generating discovery technique to profile proteins in patients with PH-HFpEF and control subjects. Followed by in-depth validation using independent human samples collected by multiple centers and characterization with in vitro cell cultures and in vivo animal models, our study identifies B2M (β2-microglobulin), a component of MHC I (major histocompatibility complex class I), as a previously unrecognized regulator of PH-HFpEF.

MATERIALS AND METHODS

Please see Expanded Methods and Supplemental Tables in the Supplemental Material for details of the material used. All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Human Subjects and Ethical Considerations

Deidentified plasma samples and muscle biopsies of subjects with PH-HFpEF participating in clinical trials (URL: https://www.clinicaltrials.gov; unique identifiers: NCT03629340 and NCT01431313) were collected at baseline within a month of confirmed diagnosis by RHC under protocols approved by the University of Pittsburgh Institutional Review Board and University of California San Francisco Institutional Review Board. Deidentified plasma samples of subjects with PH-HFpEF and patients with HFpEF without PH were collected more than a month of RHC or echocardiogram under protocols approved by the University of Illinois at Chicago Institutional Review Board. In addition, deidentified plasma samples of control subjects were collected under protocols approved by the Indiana University Institutional Review Board.

MS Experiments

Sample Preparation and Nano-Liquid Chromatography MS Analysis

Plasma samples (10 µL of each sample) were depleted of abundant plasma proteins using High Select Top14 abundant protein depletion spin columns (No. A36369; Thermo Fisher Scientific). Individual samples were then processed using EasyPep Mini MS Sample Prep Kits (No. A40006; Thermo Fisher Scientific) according to the manufacturer's procedure, which transformed protein-level samples into reduced/alkylated tryptic peptides. Samples were broken up into 5 separate batches; each batch was labeled with a separate set of tandem mass tag (TMT) 11-plex reagents (No. A37725; Thermo Fisher Scientific). Prelabeling pooled samples (≈20% of each individual sample pooled) were used as bridge channels to enable quantitative comparison across the sample batches. Following the labeling, samples in each batch were mixed and the combined group samples were each fractionated into 8 fractions by high pH reversed phase using Pierce High pH Reversed-Phase Peptide Fractionation Kit (No. 84868; Thermo Fisher Scientific). Hence, a total of 40 separate samples for LC-MS (5 batches with 8 fractions each) was prepared. Each sample (≈1 µg loaded on column) was analyzed separately by nano-liquid chromatography MS using Thermo Scientific UltiMate 3000 nanoLC system and Orbitrap Fusion Tribrid Mass Spectrometer equipped with an EasySpray source. Briefly, chromatography was performed on a 50-cm C18 nanoLC column with a 2-hour effective chromatography gradient, and MS was performed using an optimized SPS MS^3 acquisition method to attain the highest number of accurate quantifiable protein identifications.

Processing and Analysis of MS Data

Raw data files were processed using Proteome Discoverer v2.3 (Thermo Fisher Scientific), and the tandem MS data were then searched using SEQUEST algorithms against a human UniProt (Swiss-Prot only) database (released June 2019) with common contaminant proteins. The search parameters included trypsin as the protease with maximum of 2 missed cleavages allowed; oxidation of methionine (+15.9949 Da) and deamidation of asparagine and glutamine (+0.9848 Da) were set as a dynamic modification while static modifications included carbamidomethyl (+57.0215 Da) at cysteine and TMT as a static modification of lysine residues and peptide N-termini (+229.1629 Da). Precursor mass tolerance was set at 10 ppm, and fragment mass tolerance was set at 0.6 Da. Peptide confidence was estimated with the Percolator node. Peptides were filtered at q value (false discovery rate) <0.01 based on a decoy database search. Reporter ions for TMT labeled peptides were quantified using the Reporter Ions Quantifier Node included a TMT11plex quantification method in Proteome Discoverer with a peak integration tolerance of 20 ppm and an integration method based on the most confident centroid peak at the MS^3 level. Only unique peptides were used for quantification, with protein groups considered for peptide uniqueness. Peptides with an average reporter signal-to-noise ratio >10 were used for protein quantification. Correction for the isotopic impurity of reporter quantification values was applied. The normalization was performed in 2 steps. First, peptide reporter ion signal-to-noise values were normalized to the total sum per channel. Second, TMT batch effects were reduced by row-wise normalization based on median intensities. Peptides with signal-to-noise ratio below 10 were reported as missing values and were omitted from the analysis determining individual protein *P* values and fold changes. Once the normalization has been completed, the proteins.txt output file was imported into Perseus v. 1.6.13.0 software for further statistical analysis and data visualization. The Perseus output was used for generating volcano plot with the significance threshold at $P<0.05$ and an S_0 (artificial within-group variance) of 0.868. The S_0 value was determined by significance analysis of microarray procedure performed in the R software environment using the samr package.[21]

Animal Studies

B2m KO (*B2m*$^{-/-}$) and WT (wild-type) mice were purchased from The Jackson Laboratory (No. 002087 and No. 000664, respectively). Beginning at 8 weeks of age, *B2m*$^{-/-}$ and WT mice were randomly assigned to high-fat diet (HFD; 60% lipids/kcal; No. D12492; Research Diets) or regular diet (10% lipids/kcal; No. D12450J; Research Diets) exposure for 16 weeks. As female mice are protective in experimental models of PH and HFpEF,[22,23] only male mice were used in this study. *Sirt3* (sirtuin-3) floxed mice were provided by Dr Joan Auwerx and were crossed with mice expressing *Cre* recombinase under the control of a human α-skeletal actin promoter (HSA-*Cre*; No. 006149; The Jackson Laboratory) to obtain *Sirt3*$^{skm-/-}$ mice.[24] At the age of 8 weeks, *Sirt3*$^{skm-/-}$ and their littermate WT mice were fed a regular diet for 16 weeks. All animals were maintained in a normoxic environment. All experimental procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. Animal studies reported in this article are in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Hemodynamic Measurements

Mice were weighed and anesthetized with isoflurane (5% for induction, 2% during surgery, and 1% while performing pressure measurements). Right ventricular systolic pressure (RVSP) and LV end-diastolic pressure were measured using a Millar catheter. Weights of RV and LV+septum normalized to tibial length were used as indexes of ventricular mass. Blinded data analysis was performed. Success rates of invasive RVSP and LV end-diastolic pressure measurements were 90% and 80%, respectively, across experiments—values typical for this level of experimental complexity.

Statistical Analysis

Statistical analyses were performed using the Prism 10.0.2 software. Statistical comparison between 2 groups was performed using the Mann-Whitney *U* test. For in vitro studies, unpaired Student *t* test was performed after testing for normality with the Shapiro-Wilk test and equal variance. Comparison among ≥3 groups was performed using 1-way or 2-way ANOVA followed by Tukey post hoc test. If the normality tests failed (homogenous variance is satisfied), Kruskal-Wallis test followed by Dunn post hoc test was performed. For differences in blood glucose levels during the glucose tolerance test, 2-way repeated measures ANOVA followed by Bonferroni post hoc test was performed. Values of $P<0.05$ were considered statistically significant.

RESULTS

MS-Based Plasma Proteomics Identifies High Protein Abundance Levels of B2M in Patients With PH-HFpEF

Using nano-capillary liquid chromatography electrospray ionization MS and TMT isobaric labels, plasma from human patients with PH-HFpEF (n=16; Table S1) and random control subjects (n=17) were processed for a MS experiment in which plasma proteomes were characterized and quantitatively compared (Figure 1A). Overall, 602 proteins were identified across control subjects and patients with PH-HFpEF. Among them, 25 proteins were found to have statistically significant higher or lower abundance levels in patients with PH-HFpEF compared with control subjects (Figure 1B and 1C). Consistent with a previous report, protein abundance levels of a common biomarker of inflammation and cardiovascular distress, CRP (C-reactive protein),[25] were also elevated in our PH-HFpEF patient cohort. Protein abundance levels of B2M, which have been correlated with disease severity in patients with peripheral arterial disease or HF with reduced ejection fraction,[26–28] were also increased in patients with PH-HFpEF. Elevated circulating levels of B2M have also been implicated in various cancers, pulmonary fibrosis, atherosclerosis, and aging.[29–32] Moreover, compared with the control subjects, protein abundance levels of SAA1 (serum amyloid A1), a major acute-phase protein mediating inflammatory amyloidosis in HFpEF, CHGA (chromogranin-A), a neurohumoral factor that predicts mortality in HF, and CA3 (carbonic anhydrase 3), which associates with dilated cardiomyopathy in HF, were elevated in patients with PH-HFpEF.[33,34] In contrast, we did not observe any significant change in protein abundance of galectin-3, an inflammation marker that has been associated with PH-HFpEF pathogenesis,[35] in our study cohort. Given that B2M is a highly abundant protein in plasma, making it an attractive candidate for sensitive detection and accurate quantitative analysis, and that it is 1 of the 3 differentially expressed proteins, along with CRP and BTBD16 (BTB/POZ [broad complex Tramtrack bric-a-brac/Pox virus and zinc finger] domain-containing protein 16), detected in all of our samples, considering its reported association with various cardiac and pulmonary diseases, we selected B2M for further validation.

Plasma B2M Levels Are Significantly Elevated in Patients With PH-HFpEF and Are Associated With Disease Severity

We measured B2M levels by ELISA in plasma obtained from a validation cohort of patients with PH-HFpEF(n=15; age, 64±11.1 years; without co-occurring kidney dysfunction, confirmed diagnosis by RHC of a resting mean pulmonary artery pressure [mPAP] ≥25 mm Hg, pulmonary capillary wedge pressure [PCWP] ≥15 mm Hg, and transpulmonary pressure gradient ≥12 mm Hg OR during exercise mPAP >30 mm Hg, PCWP ≥20 mm Hg, and total pulmonary resistance ≥3[36]; Table S2), patients with HFpEF without PH (n=9; age, 55.4±17.9 years; mPAP ≤20 mm Hg by RHC or tricuspid regurgitation velocity ≤3 m/s measured by echocardiogram), and random control subjects free of HF, PH, chronic kidney disease, cancers, and a few other rare diseases (n=44; age, 65.5±7.2 years). In agreement with the results obtained from proteomic analysis (Figure 1D), ELISA assays demonstrated that circulating concentrations of B2M were higher in the validation cohort of patients with PH-HFpEF, especially in patients with diagnosis confirmed by resting hemodynamics, compared with patients with HFpEF without PH or control subjects (Figure 2A). Importantly, we found a positive correlation between plasma B2M levels and resting mPAP (r=0.6246; $P=0.0333$) in patients with PH-HFpEF whose plasma samples were collected within a month of RHC (Figure 2B). A positive correlation between plasma B2M levels with resting PCWP (r=0.6667; $P=0.0209$), transpulmonary pressure gradient (r=0.607; $P=0.0397$), pulmonary vascular resistance (r=0.5944; $P=0.0457$), or right atrial pressure (RAP; r=0.8642; $P=0.0005$) was also observed (Figure 2C through 2F). We did not observe any difference between plasma B2M levels and systemic vascular resistance, cardiac output, cardiac index, or LV ejection fraction in patients with PH-HFpEF (Figure S1). In addition, no significant correlation between plasma B2M levels and exercise mPAP or PCWP was observed (Figure S2). Of note, circulation concentrations of B2M were positively correlated with NT-proBNP (r=0.5964; $P=0.0213$) in patients with PH-HFpEF (Figure S3A). However, no significant positive correlation between plasma NT-proBNP levels and resting mPAP, resting PCWP, transpulmonary pressure gradient, pulmonary vascular resistance, or RAP was observed (Figure S3B through S3F).

Skeletal Muscle Levels of B2M Are Increased in Mice With Skeletal Muscle SIRT3 Deficiency or HFD-Induced PH-HFpEF

Our group has previously reported that defects in skeletal muscle SIRT3, the mitochondrial deacetylase

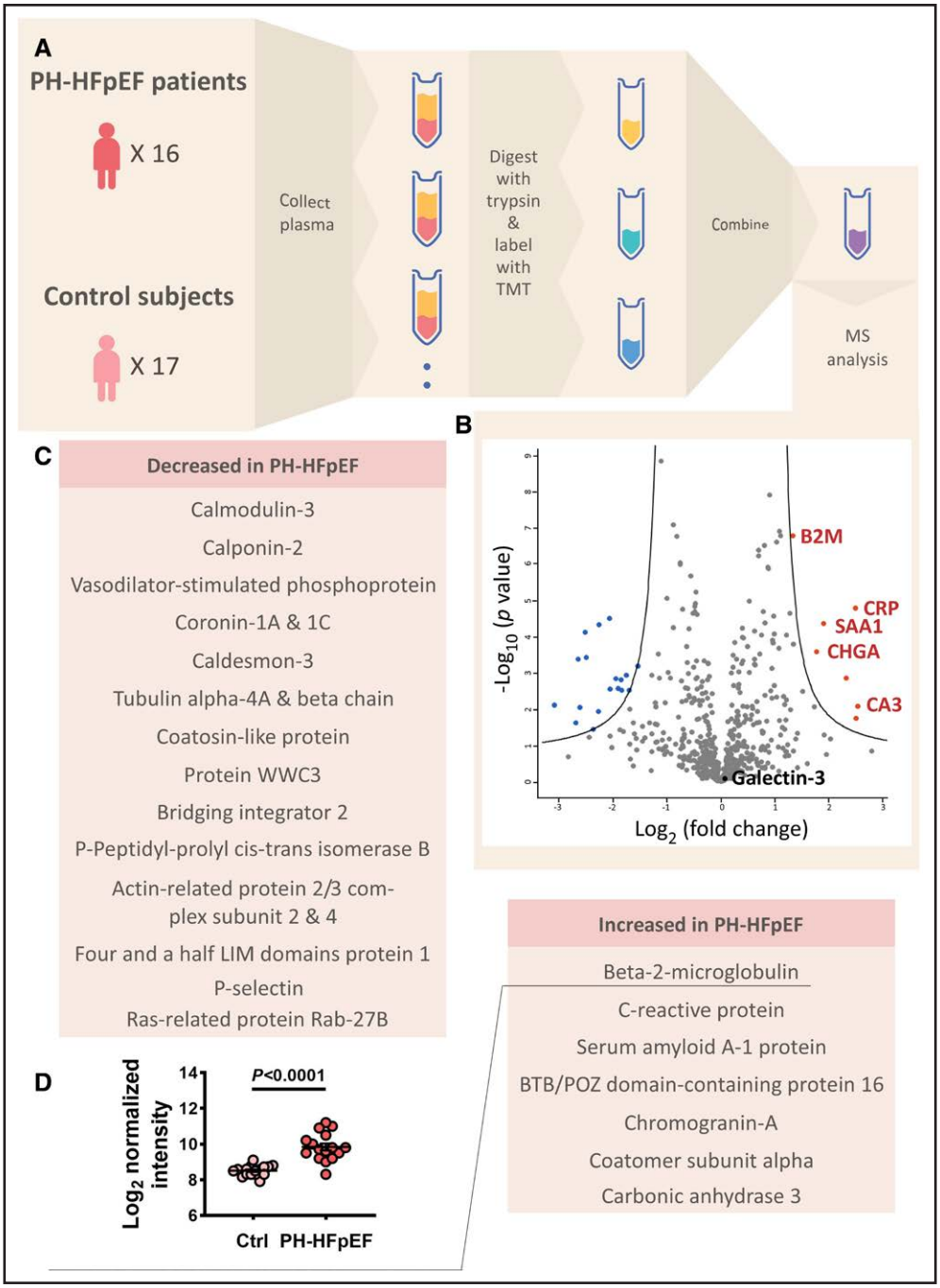

Figure 1. Changes in plasma protein abundance profiles in patients with pulmonary hypertension (PH)–heart failure with preserved ejection fraction (HFpEF).

A, Schematic overview of mass spectrometry (MS)–based plasma proteomic analysis. **B**, Volcano plot showing the fold change and statistical significance (*P* value was determined using the Student *t* test) for protein abundance levels. The solid curves represent the significance threshold at $P<0.05$ and an S_0 of 0.868. **C**, Changing protein abundances in PH-HFpEF (age, 69.8±7.9 years; male sex, 8; body mass index, 39.1±10.4; mean pulmonary artery pressure [mPAP], 39.4±8.1 mm Hg; pulmonary capillary wedge pressure [PCWP], 20.2±4.5 mm Hg; World Health Organization function class II: 1 [6%], III: 14 [88%], and IV: 1 [6%]). **D**, Relative protein abundance of B2M (β2-microglobulin). Data are mean±SEM. *P* value was determined by Mann-Whitney *U* test. CA3 indicates carbonic anhydrase 3; CHGA, chromogranin-A; CRP, C-reactive protein; SAA1, serum amyloid A1; TMT, tandem mass tag; and WWC3, WW and C2 domain containing protein 3.

that is critical for modulating metabolic syndrome, PH, and aging,[37–39] are associated with increased pulmonary vascular remodeling and pulmonary pressures in an Ob-Su rat model of PH-HFpEF.[17] To evaluate whether skeletal muscle SIRT3 deficiency affects induction and secretion of B2M, we measured skeletal muscle expression and circulating B2M levels by Western blot and ELISA, respectively, in soleus

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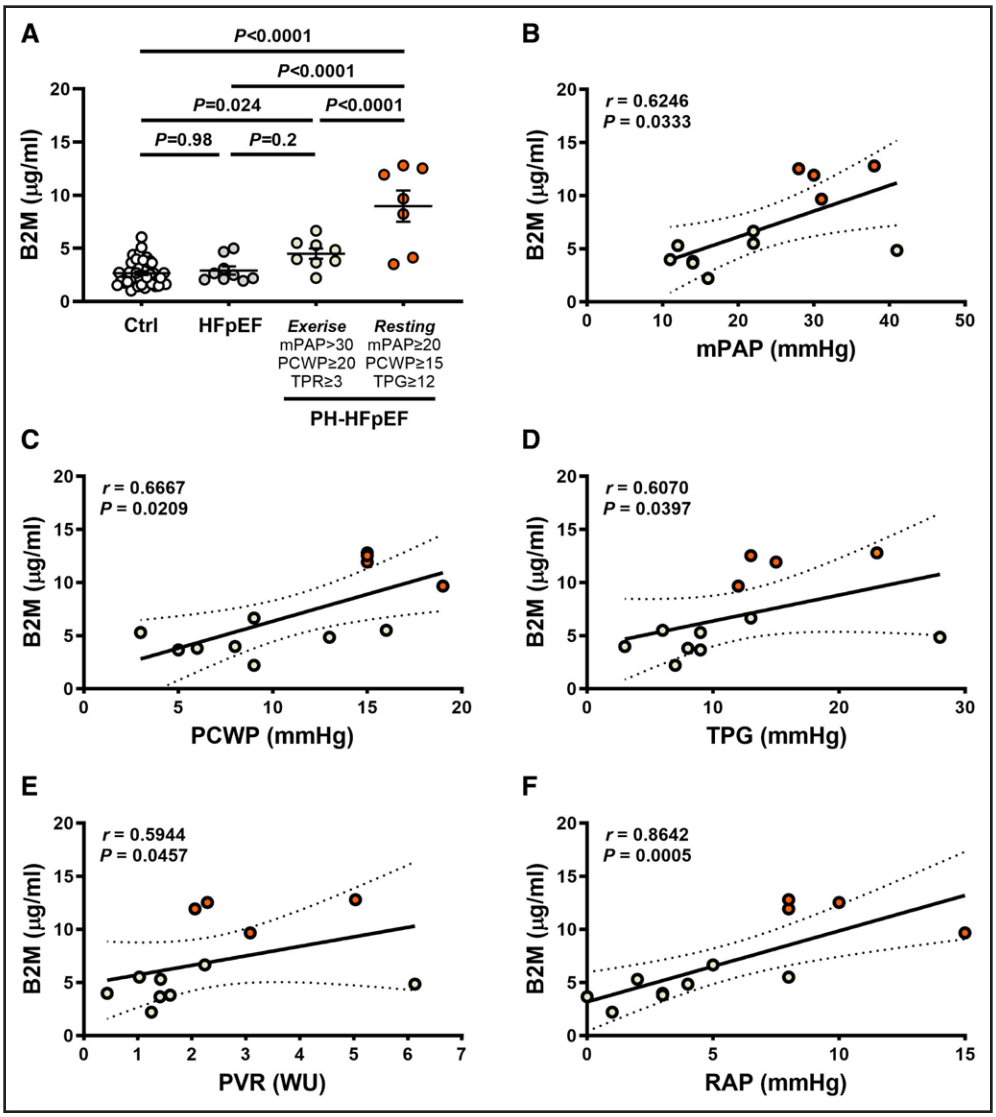

Figure 2. Circulating levels of B2M (β2-microglobulin) are elevated in patients with pulmonary hypertension (PH)–heart failure with preserved ejection fraction (HFpEF).
A, Circulating levels of B2M were measured by ELISA in plasma of the validation cohort of control (Ctrl) subjects, HFpEF patients without PH, and PH-HFpEF patients (diagnosis is confirmed by right heart catheterization [RHC] when the resting mean pulmonary artery pressure [mPAP] was ≥25 mm Hg, pulmonary capillary wedge pressure [PCWP] was ≥15 mm Hg, and transpulmonary pressure gradient [TPG] was ≥12 mm Hg or during exercise mPAP was >30 mm Hg, PCWP ≥20 mm Hg, and total pulmonary resistance [TPR] ≥3). Data are mean±SEM. *P* value was determined using 1-way ANOVA followed by Tukey post hoc test. **B** through **F**, Correlation between circulating levels of B2M and resting mPAP (**B**), PCWP (**C**), TPG (**D**), pulmonary vascular resistance (PVR; **E**), or right atrial pressure (RAP; **F**) in patients with PH-HFpEF whose plasma samples were collected within a month of RHC. Spearman r is shown. The upper and lower dotted lines represent the 95% CIs of the regression.

and plasma obtained from skeletal muscle–specific *Sirt3* KO mice (*Sirt3*$^{skm-/-}$). Our data showed that skeletal muscle expression levels of B2M were higher in *Sirt3*$^{skm-/-}$ mice compared with WT mice (Figure 3A). B2M levels were significantly higher in plasma obtained from *Sirt3*$^{skm-/-}$ mice, which positively correlated with elevated RVSP (Figure 3B; Figure S4A). Subsequently, we measured skeletal muscle expression and circulating levels of B2M in mice fed an HFD, which reduces SIRT3 in skeletal muscle and induces a metabolic syndrome–associated PH-HFpEF phenotype.[40–42] Our data showed that skeletal muscle expression and plasma concentration of B2M levels were increased in HFD-exposed mice (Figure 3C and 3D). A positive correlation between plasma B2M levels and RVSP was also observed in HFD-exposed mice (Figure S4B). Of note, plasma levels of other skeletal muscle–derived immune molecules, such as IL-6 and IL-15,[43,44] remained unchanged in *Sirt3*$^{skm-/-}$ and HFD-exposed mice (Figure S5). As B2M is reabsorbed and catabolized by the kidney,[30] enhanced B2M expression was observed in the

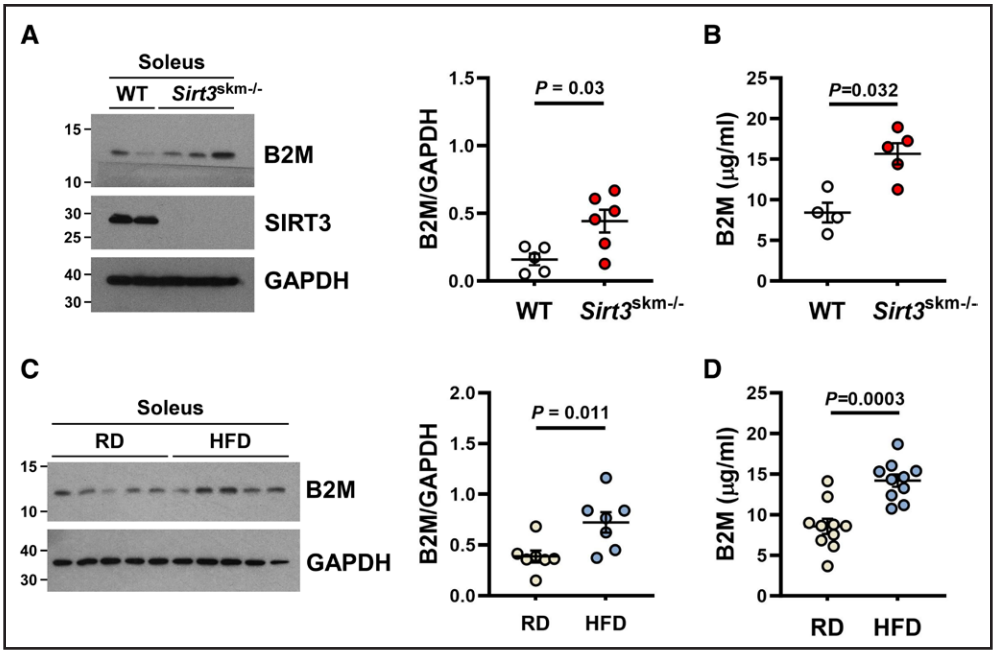

Figure 3. Skeletal muscle levels of B2M (β2-microglobulin) are increased in mice with skeletal muscle SIRT3 (sirtuin-3) deficiency or high-fat diet (HFD)–induced pulmonary hypertension (PH)–heart failure with preserved ejection fraction (HFpEF). **A** and **B**, Skeletal muscle (**A**) and plasma (**B**) levels of B2M in WT (wild-type) and skeletal muscle–specific SIRT3 knockout (*Sirt3*$^{skm-/-}$) mice. **C** and **D**, Skeletal muscle (**C**) and plasma (**D**) levels of B2M in mice fed a regular diet (RD; 10% lipids/kcal) or HFD (60% lipids/kcal) for 16 weeks. Data are mean±SEM. *P* value was analyzed by Mann-Whitney *U* test.

kidney of HFD-exposed mice (Figure S6A). However, B2M protein expression was unchanged in the LV, RV, adipose tissue, lung, liver, and spleen after 16 weeks of HFD exposure (Figure S6B through S6G). No difference of B2M expression levels between RV and LV, after 16-week exposure to regular diet or HFD, was observed (Figure S6H). Together, these data suggest the link between skeletal muscle SIRT3 deficiency and the induction/secretion of B2M.

Skeletal Muscle Expression Levels of B2M Are Increased in PH-HFpEF Patients and Correlated Significantly With Resting PCWP and RAP

To further assess the clinical relevance of our findings, we measured B2M expression levels in muscle biopsies from patients with PH-HFpEF and control subjects free of HF and PH. As shown in Figure 4A, muscle biopsies of patients with PH-HFpEF had higher protein expression levels of B2M and lower activation levels of SIRT3 (the antibody we used specifically recognizes the short active form of SIRT3 at ≈28 kDa, which contains the catalytic domain and regulates deacetylation) compared with that of control subjects. Notably, skeletal muscle B2M expression levels significantly correlated with resting PCWP (r=0.8619; *P*=0.0044; Figure 4B) and resting RAP (r=0.7197; *P*=0.0338; Figure 4C), demonstrating an association and clinical relevance of skeletal muscle B2M in PH-HFpEF.

Treatment With B2M Increases Pulmonary Arterial Vascular Endothelial Cell Migration/Proliferation and Promotes Pulmonary Arterial Vascular Smooth Muscle Cell Proliferation

While B2M has been associated with pulmonary fibrosis, emphysema, cardiac fibroblast activation, atherosclerosis, and aging,[29,31,32,45,46] it is currently unknown whether B2M can regulate pulmonary vascular remodeling. To assess whether B2M affects endothelial abnormalities and medial wall thickness, we administered exogenous B2M to cultured human pulmonary arterial vascular endothelial cells and pulmonary arterial vascular smooth muscle cells (PAVSMCs). Our data showed that B2M exposure increased cell migration and proliferation in pulmonary arterial vascular endothelial cells (Figure 5A and 5B). Similarly, our data showed that administration of B2M increased cell proliferation in correlation with elevated expression levels of PCNA (proliferating cell nuclear antigen) in PAVSMCs (Figure 5C and 5D). Collectively, our findings suggest a pathogenic role of B2M in the regulation of pulmonary vascular remodeling.

B2M Whole-Body KO Mice Are Protected From HFD-Induced PH-HFpEF

To evaluate the impact of B2M on metabolic syndrome–associated PH-HFpEF, *B2m*$^{-/-}$ and WT mice were exposed to HFD for 16 weeks (Figure 6A). Consistent

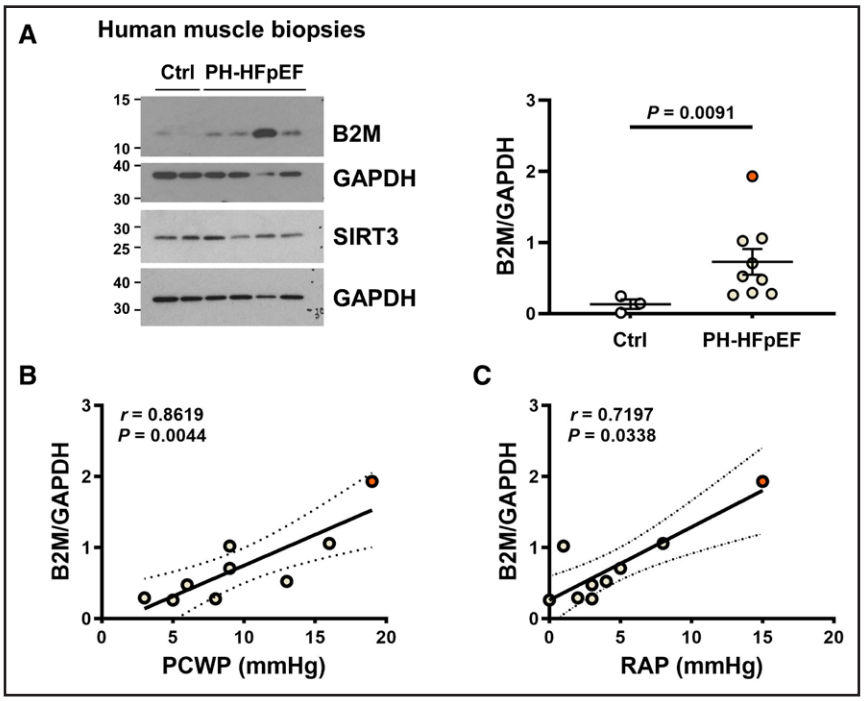

Figure 4. Muscle expression levels of B2M (β2-microglobulin) are increased in patients with pulmonary hypertension (PH)–heart failure with preserved ejection fraction (HFpEF). **A**, B2M expression levels were measured in muscle biopsies of patients with PH-HFpEF and control subjects by Western blots. Data are mean±SEM. *P* value was analyzed by Mann-Whitney *U* test. **B** and **C**, Correlation between skeletal muscle B2M expression with resting pulmonary capillary wedge pressure (PCWP; **B**) or right atrial pressure (RAP; **C**). Spearman r is shown. The upper and lower dotted lines represent the 95% CIs of the regression.

with our previous observations, HFD-exposed WT mice exhibited significantly higher body weights, glucose intolerance, RVSP, LV end-diastolic pressure, biventricular hypertrophy, cardiac index, and RV contractility than regular diet–exposed WT mice (Figure 6B through 6G; Figure S7). While no difference in body weights, LV hypertrophy, LV end-diastolic pressure, or cardiac index was observed, HFD-exposed *B2m*$^{-/-}$ mice exhibited significantly improved glucose intolerance, lowered RVSP, decreased RV contractility, and reduced RV hypertrophy compared with WT mice fed an HFD (Figure 6B through 6G; Figure S7). In addition, HFD-exposed *B2m*$^{-/-}$ mice had a lower percentage of wall thickness in comparison with HFD-exposed WT mice (Figure 6H). We did not observe any difference related to systemic vascular remodeling in *B2m*$^{-/-}$ and WT mice (Figure S8). Correspondingly, improvement of pulmonary vascular remodeling was associated with lower PCNA expression in PAVSMCs of HFD-exposed *B2m*$^{-/-}$ mice (Figure 6I). Taken together, these data highlight the potential benefit of reduced B2M on metabolic syndrome–associated PH-HFpEF.

DISCUSSION

The main findings of the present study include: (1) discovery-based untargeted plasma proteomic analysis–identified high protein abundance levels of B2M in patients with PH-HFpEF; (2) skeletal muscle SIRT3 deficiency is associated with induction and secretion of B2M in animal models and human subjects with PH-HFpEF; (3) circulating and skeletal muscle expression levels of B2M correlate with PH-HFpEF severity; (4) B2M increases pulmonary arterial vascular endothelial cell migration/proliferation and promotes PAVSMC proliferation; and (5) loss of B2M improves metabolic syndrome–associated PH-HFpEF. These findings not only reveal a previously unknown pathogenic role for B2M in PH-HFpEF but also suggest the potential of using circulating and skeletal muscle B2M levels as biomarkers for PH-HFpEF.

B2M comprises the light chain of MHC I that forms an active part of the adaptive immune system. As it is noncovalently associated with the light chain and has no direct attachment to the cell membrane, free B2M circulates in the blood as a result of shedding or intracellular release.[27] The net concentration of B2M is determined by its generation/secretion into circulation and its elimination by the kidneys.[30] As such, in people without kidney disease, elevated B2M has been considered as a marker of altered cell proliferation.[30] Increased circulating levels of B2M have been implicated in various cancers and associated with cancer progression with poor prognosis.[30,47] B2M has also been associated with increased colorectal cancer risk.[30] Additionally, there is evidence to correlate elevated serum B2M concentrations with the development and progression of pulmonary fibrosis in patients with chronic obstructive pulmonary disease and emphysema.[32,45] B2M was also found to be associated with adverse cardiovascular outcomes in patients with asymptomatic carotid atherosclerosis.[31] Furthermore, plasma proteome studies found B2M to be a risk marker for coronary heart disease in postmenopausal women and to correlate with disease severity in patients with peripheral arterial disease or HF with reduced ejection fraction.[26–28] Our data show that B2M is present at higher levels in patients with PH-HFpEF. Of note, patients

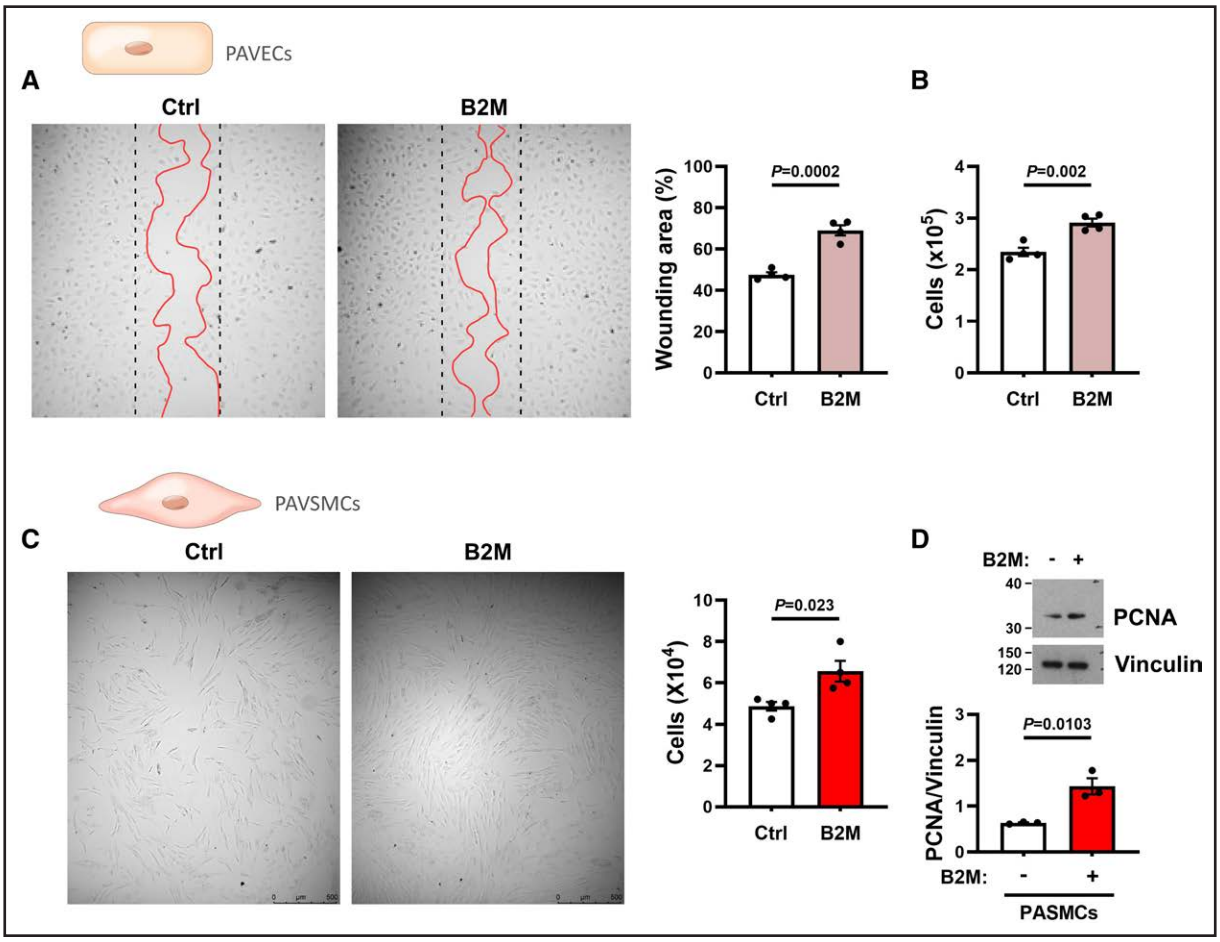

Figure 5. Treatment with B2M (β2-microglobulin) induces pulmonary arterial vascular endothelial cell (PAVEC) migration/proliferation and promotes pulmonary arterial vascular smooth muscle cell (PAVSMC) proliferation.
A and **B**, Human PAVECs were administered with exogenous B2M (10 μg/mL) for 4 days. Representative images of cell migration and related quantitative data (**A**). Cell proliferation assessed by cell counts (**B**). **C** and **D**, Human PAVSMCs were exposed to B2M (10 μg/mL) for 5 days. Representative images of cell numbers and cell proliferation assessed by cell counts (**C**). Representative Western blots for PCNA (proliferating cell nuclear antigen) protein expression levels (**D**). Data are mean±SEM. *P* value was determined using unpaired Student *t* test after testing for normality with Shapiro-Wilk test and equal variance.

with diagnosis confirmed by resting hemodynamics have higher circulating B2M levels than those with diagnosis confirmed by exercise hemodynamics, the latter of which unmasks patients without overt PH or HFpEF. When analyzing clinical data, we observe a significant correlation between increased circulating B2M and pulmonary hemodynamics, such as PCWP, mPAP, transpulmonary pressure gradient, pulmonary vascular resistance, and RAP, at rest, but not during exercise, in patients with PH-HFpEF. These findings suggest that B2M is probably related to chronic, rather than acute, alterations of pulmonary hemodynamics in HFpEF. These findings also suggest that elevated B2M may capture chronic pathological changes in patients with pulmonary vascular dysfunction–HFpEF phenotype (combined precapillary and postcapillary PH), from passive hypertension with chronic increases in LV filling pressure, pulmonary vascular dysfunction/cellular remodeling, and elevated pulmonary pressures, to right heart dysfunction. Whether circulating B2M levels can be a useful noninvasive tool to define/refine specific patient subphenotypes and to identify patients in a high-throughput fashion requires further investigations.

Despite the reports implicating increased circulating B2M concentrations in cancers, pulmonary fibrosis, emphysema, aging, and HF with reduced ejection fraction, little is known about the mechanism by which B2M contributes to these diseases. Beyond being a simple biomarker, exogenous B2M injection has been shown to promote age-related cognitive dysfunction and impaired neurogenesis via cell surface expression of MHC I.[29] B2M has also been shown to promote cancer cell growth and metastasis through increased inflammatory activities and activation of PI3K/Akt (phosphoinositide 3-kinase/protein kinase B), ERK (extracellular signal-regulated kinase), or NLRP3 (NOD-like receptor protein 3) inflammasomes.[47–49] In addition, cardiomyocyte-secreted B2M contributes to fibroblast activation after ischemia injury and platelet-derived B2M induces monocyte proinflammatory

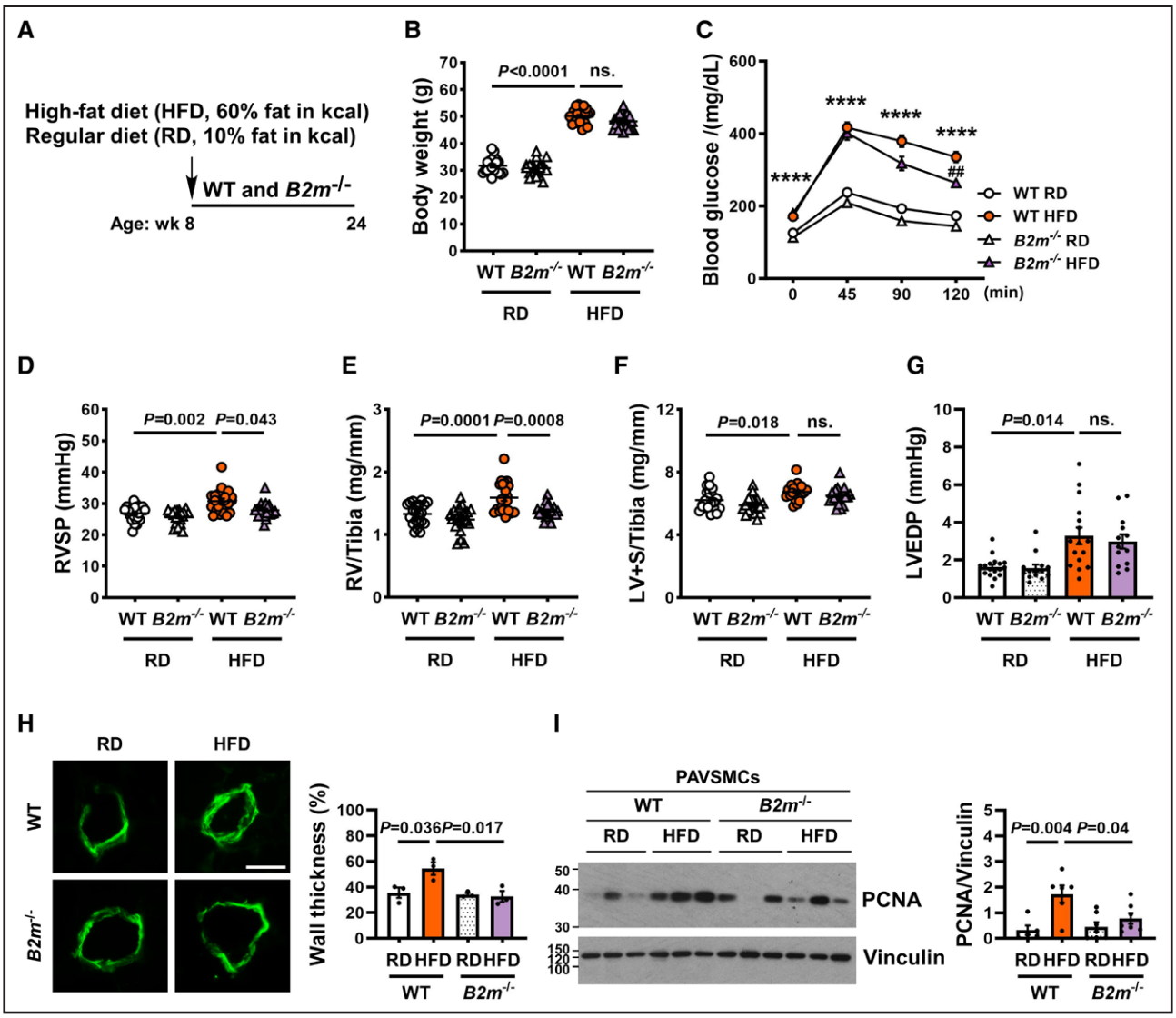

Figure 6. B2M (β2-microglobulin) whole-body KO (knockout) mice protect against metabolic syndrome–associated pulmonary hypertension (PH)–heart failure with preserved ejection fraction (HFpEF).

A, Eight-week-old WT (wild-type) and whole-body B2M KO mice (*B2m*$^{-/-}$) were fed an regular diet (RD) or high-fat diet (HFD) for 16 weeks. **B** through **G**, At week 16, body weights (**B**), glucose tolerant abilities (**C**), right ventricular systolic pressures (RVSP; **D**), and left ventricular end-diastolic pressure (LVEDP; **G**) were measured. **E** and **F**, Weights of right ventricle (RV; **E**) and left ventricle (LV)+septum (S; **F**) normalized to tibial length were used as an index of ventricular hypertrophy. **H**, Representative images of lung sections stained with α-SMA (α-smooth muscle actin; 40×) and quantification of wall thickness from the mean of 5 to 6 vessels per lung section from 3 mice per group. Scale bar, 30 μm. **I**, PCNA (proliferating cell nuclear antigen) levels were analyzed by Western blot in pulmonary arterial vascular smooth muscle cells (PAVSMCs) of *B2m*$^{-/-}$ and WT mice. Data are mean±SEM. *P* value was determined using 2-way ANOVA followed by Tukey post hoc test. For Figure 6D and 6G, *P* value was determined using the Kruskal-Wallis test followed by Dunn post hoc test. For glucose tolerance test, 2-way repeated measures ANOVA followed by Bonferroni post hoc test was performed. ****$P<0.0001$ WT HFD vs WT RD, and $^{\#\#}P<0.01$ *B2m*$^{-/-}$ HFD vs WT HFD.

differentiation,[46,50,51] highlighting a potential role of B2M as a signaling factor. Our in vitro and in vivo studies indicate a pathogenic role of B2M in the regulation of pulmonary vascular proliferative remodeling. As elevated circulating concentration of B2M is associated with increased protein expression of B2M in the skeletal muscle, our data further suggest B2M may act as an endocrine signaling molecule to induce pulmonary vascular remodeling via increasing pulmonary arterial vascular endothelial cell migration/proliferation and PAVSMC proliferation.

While our data show that skeletal muscle expression levels of B2M correlate with PH-HFpEF severity, little is known about the pathological mechanism underlying increased skeletal muscle B2M in PH-HFpEF. Upregulation and prolonged expression of MHC I in muscle cells is a hallmark of inflammatory myopathies, which have been recognized as one of the extracardiopulmonary sources of inflammation in HFpEF and PH.[52–55] Proinflammatory cytokines increase surface expression of MHC I in muscle cells.[52] Oxidative stress and mitochondrial

dysfunction have also been linked to inflammatory myopathies.[56] Here, we show that SIRT3 deficiency and HFD exposure, both of which are known to induce reactive oxygen species production, mitochondrial dysfunction, and chronic metabolic inflammation, increase skeletal muscle B2M expression. SIRT3 has been recognized as an important regulator of skeletal muscle metabolism.[57] Loss of SIRT3 induces oxidative stress, which inhibits protein synthesis and promotes protein degradation, and has been shown to accelerate angiotensin II–induced skeletal muscle atrophy.[57,58] In line with previous findings that HFD leads to muscle atrophy,[59,60] decreased muscle fiber cross-sectional area was also observed in HFD-exposed mice (Figure S9). Reduced skeletal muscle function and muscle atrophy have been associated with exercise intolerance in patients with HFpEF.[61,62] Impaired skeletal function has also been consistently observed in patients with PH.[63] As SIRT3 activation is reduced, accompanied by increased expression of B2M, in the skeletal muscle of patients with PH-HFpEF, it is possible that diminished SIRT3 activation in the skeletal muscle contributes to elevated expression of B2M in PH-HFpEF. In addition, it has been reported that IFN-γ (interferon-γ) induces the production and secretion of B2M in lymphocytes.[64] Platelet-derived B2M has also been shown to regulate monocyte inflammatory responses.[50] Since elevated circulation concentrations of IFN-γ and enhanced platelet respiratory capacity have been associated with adverse clinical outcomes and RV dysfunction in PH-HFpEF,[65,66] it remains to be clarified whether platelets, monocytes, and lymphocytes can influence circulation B2M levels in PH-HFpEF. Future studies are also needed to determine whether skeletal muscle B2M contributes to PH-HFpEF pathogenesis and the interplay of abovementioned molecular, immunologic, and metabolic processes in this context.

Management of PH-HFpEF is challenging due to the lack of proven PH therapies in the setting of HFpEF. SGLT2 inhibitors were recently approved for the treatment of HFpEF.[3] SGLT2 inhibitors have been reported to improve exercise-induced PH in Ob-Su rats and patients with type 2 diabetes and reduce PA pressure in patients with HF[4–6]; however, the search for effective therapies for PH-HFpEF remains. Using an HFD model of metabolic syndrome–associated PH-HFpEF, we demonstrate that loss of B2M exhibits beneficial effects on glucose intolerance, pulmonary vascular proliferative remodeling, PH, and RV hypertrophy. This highlights B2M as a promising therapeutic candidate for the treatment of PH-HFpEF. More work is needed to determine whether B2M affects other cell types in PH-HFpEF pathogenesis and to evaluate whether B2M inhibition can reduce or reverse PH-HFpEF in the more severely affected animal models.[17,18] As skeletal muscle SIRT3 was found to be decreased in the rat model of pulmonary arterial hypertension (group 1 PH) and SIRT3-deficient mice develop spontaneous pulmonary arterial hypertension,[38,67] the role of B2M in the pathogenesis of pulmonary arterial hypertension merits future investigations. In addition, high B2M levels are associated with lung emphysema and pulmonary fibrosis development in patients with chronic obstructive pulmonary disease.[32,45] B2M has also been shown to complex with HFE (hemochromatosis gene) protein to activate iron metabolism, such as HIF-1α (hypoxia-inducible factor 1α) signaling, which induces epithelial cells to mesenchymal transition.[45,68] As hypoxia, inflammation, and loss of capillaries in severe emphysema are associated with PH development and pulmonary vascular remodeling in chronic obstructive pulmonary disease (group 3 PH),[69] our findings may open a new avenue for approaches targeting B2M in the management of PH development and pulmonary vascular remodeling in chronic obstructive pulmonary disease as well.

We recognize several limitations in the present study. First, human plasma samples were collected by multiple centers, with various diagnostic methods, different blood-drawn RHC period, and limited sample size. Second, the age of individuals among random control subjects and patients with PH-HFpEF was not compatible in the discovery-based untargeted plasma proteomic analysis. However, the observation of higher circulating levels of B2M in PH-HFpEF is supported by age-matched controls in the validation study. Third, we did not evaluate the impact of comorbidities, other than chronic kidney disease, race, and background therapies in the current study. Fourth, our investigation of skeletal muscle B2M levels was limited due to low sample size of age-matched control subjects. Large-scale validation studies with well-controlled subject selection, diagnosis criteria, and sample collection period are needed in the future.

In conclusion, our studies suggest the potential of using circulating and skeletal muscle B2M levels as biomarkers for PH-HFpEF to guide future research toward the facilitation of screening, diagnosis, refinement of specific patient phenotype, and identification of patients in a more efficient manner. Our findings also provide new insights into the mechanistic basis of B2M in the regulation of pulmonary vascular proliferative remodeling and PH-HFpEF. From a translational perspective, our data raise the possibility of B2M becoming an important therapeutic target for the treatment of PH-HFpEF in the future.

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Disclosures

None.

Supplemental Material

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Tables S1–S3
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