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Original article

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The angiostatic peptide endostatin enhances mortality risk prediction in pulmonary arterial hypertension

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C.E.S., A.D.E., R.L.D., and P.M.H. designed the study; S.B. and J.Y. performed the experiments and interpreted the results; C.E.S., M.G., J.Y., M.K.N., M.W.P., E.D.A., D.D. I., W.C.N. performed data collection, maintenance, and analysis; C.E.S., L.J.M., and R.D.V. performed statistical analyses; C.E.S. drafted the manuscript; all authors critically revised the manuscript for important intellectual content and approved the final version; P.M.H. and R.L.D. had access to all the data in the study and take full responsibility for the integrity and accuracy of the work.

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Abstract

Currently available noninvasive markers for assessing disease severity and mortality risk in pulmonary arterial hypertension (PAH) are unrelated to fundamental disease biology. Endostatin, an angiostatic peptide known to inhibit pulmonary artery endothelial cell migration, proliferation, and survival *in vitro*, has been linked to adverse hemodynamics and shortened survival in small PAH cohorts. This observational cohort study sought to assess 1) the prognostic performance of circulating endostatin levels in a large, multicenter PAH cohort, and 2) the added value gained by incorporating endostatin into existing PAH risk prediction models.

Endostatin ELISAs were performed on enrollment samples collected from 2,017 PAH subjects with detailed clinical data, including survival times. Endostatin associations with clinical variables, including survival, were examined using multivariable regression and Cox proportional hazards models. Extended survival models including endostatin were compared to null models based on the REVEAL risk prediction tool and ESC/ERS low risk criteria using likelihood ratio tests, Akaike and Bayesian information criteria, and C-statistics.

Higher endostatin was associated with higher right atrial pressure, mean pulmonary arterial pressure, and pulmonary vascular resistance and with shorter six-minute walk distance (p <0.01). Mortality risk doubled for each log-higher endostatin (hazard ratio 2.3, 95% confidence interval 1.6 to 3.4, p <0.001). Endostatin remained an independent predictor of survival when incorporated into existing risk prediction models. Adding endostatin to REVEAL-based and ESC/ERS criteria-based risk assessment strategies improved mortality risk prediction.

Endostatin is a robust, independent predictor of mortality in PAH. Adding endostatin to existing PAH risk prediction strategies improves PAH risk assessment.

Introduction

Pulmonary arterial hypertension (PAH) results from a complex interplay of dysregulated biological pathways, with uncontrolled cellular proliferation, inflammation, autoimmunity, and impaired angiogenesis contributing to pulmonary vascular remodeling, right ventricular (RV) failure and death (1-3). The selection and timing of clinical interventions hinges upon serial assessment of disease severity and mortality risk based on a variety of risk prediction strategies. The clinical tools currently available for risk prediction are imperfect, however. Definitive assessment of pulmonary pressures can only be made with right heart catheterization (RHC), which is invasive. Common noninvasive tests suffer a variety of limitations: six-minute walk distance (6MWD) may be confounded by musculoskeletal comorbidities; pulmonary function testing requires consistent patient effort; and echocardiography correlates poorly with invasive hemodynamics, with some echocardiographic measures qualitatively assessed and subject to interpretation.

Serum biomarkers are objective, noninvasive, and easily obtained, but to date, only the brain natriuretic peptide (BNP) and its N-terminal prohormone (NTproBNP) are commonly used in clinical practice for PAH risk stratification. While other serum biomarkers have been robustly associated with the presence of PAH and with clinical outcomes (4, 5), all have intrinsic limitations regarding their specificity for PAH or the RV, as most PAH markers have been studied due to their known relevance in left heart disease or in autoimmune or inflammatory conditions. RV dysfunction is the major determinant of morbidity and mortality in PAH (3, 6). Therefore, defining unique noninvasive markers that reflect fundamental PAH or RV pathobiology could allow for more tailored risk assessments.

Endostatin (ES) is a circulating angiostatic peptide derived from the protein collagen XVIII, alpha 1 (COL18A1) that is known to inhibit tumor angiogenesis and coronary collateral formation (7-10). Previous work by our group has demonstrated that ES inhibits pulmonary artery endothelial cell proliferation and migration and promotes endothelial cell apoptosis, fundamental features driving PAH pathobiology (11). The RV response to increased load imposed by pulmonary vascular change is the major determinant of morbidity and mortality in PAH (3, 6). Decreased angiogenesis is a feature of RV failure in preclinical models of disease (12), and decreased RV perfusion is observed in clinical PAH (13). Thus, ES may be molecularly relevant to both PAH pathogenesis and RV dysfunction, positioning ES as a potential mechanistic biomarker.

Previously published associations between circulating ES levels and survival in PAH have been inconsistent, losing significance with adjustment for established disease severity markers, including 6MWD, New York Heart Association Functional Class (NYHA FC), and NTproBNP (14). However, prior studies were conducted in small single-center cohorts, limiting multivariable modeling or subgroup analyses. Therefore, the true clinical potential of ES as a prognostic biomarker in PAH remains uncertain. In the current study, we examine the prognostic performance of ES in PAH in a large, multi-center cohort of PAH subjects. We also sought to assess the value gained by adding ES measurements to established PAH risk stratification models. We hypothesized that ES would significantly associate with PAH severity, independently predict mortality risk, and add value to existing PAH risk prediction models.

Methods

This observational cohort study was approved by the Johns Hopkins University Institutional Review Board. Serum samples and clinical data from adult subjects with PAH were obtained from the multicenter National Heart, Lung, and Blood Institute-sponsored PAH Biobank (www.pahbiobank.org). Subjects provide informed consent and serum samples at the time of enrollment. Methods for data collection and processing serum samples have been previously published (4, 5). An electrochemiluminescence assay was developed to measure ES levels on a 96 well plate from Meso Scale Discovery (Gaithersburg, MD). The full assay protocol has been previously published (5). The percent coefficient of variation for ES across plates (N=25) was 2.38%.

Cohort demographics and clinical characteristics were summarized with descriptive statistics. To examine associations between ES and clinical phenotypes, ES served as an independent variable in regressions of continuous clinical variables with adjustments for age and sex. ES levels were right-skewed and natural log-transformed to reduce the influence of outliers. To examine associations of mortality with ES dichotomized above versus below its median, unadjusted survival analyses were conducted using the Kaplan-Meier method, and adjusted analyses were performed using multivariable Cox proportional hazard models with potential confounders of the relationship between ES and survival included as covariates. Covariates were examined for collinearity using variance inflation factors and Pearson correlation coefficients; highly collinear covariates were excluded. Biomarker associations were examined in the overall cohort and in prespecified subgroups of the two predominant PAH subtypes. To assess the value of adding ES to existing noninvasive markers and established risk

prediction models, extended time-to-event models including ES were compared to null models without ES using likelihood ratio tests, Akaike and Bayesian information criteria, and C-statistics. Existing risk prediction tools tested as null or base models included the REVEAL 2.0 risk model developed from the Registry to Evaluate Early and Long-term PAH Disease Management and the French Pulmonary Hypertension Registry risk assessment strategy of tabulating the number of low-risk criteria present according to 2015 European Society of Cardiology/European Respiratory Society (ESC/ERS) guidelines (15-17). Only subjects with sufficient data available to calculate REVEAL 2.0 risk scores and tabulate ESC/ERS low risk criteria were included in assessments of the respective risk prediction tools. Missing data was considered missing not completely at random, and complete-case analyses were initially performed, followed by additional sensitivity analyses excluding variables with significant missingness. A p value of <0.05 was considered statistically significant.

Results

The cohort consisted of 2,017 subjects with PAH and was composed primarily of women (80% female) of European ancestry (EA) (82% EA) in the sixth decade of life. Subjects in this prevalent cohort had moderately severe disease, with 45% of the cohort classified as having NYHA FC III or IV symptoms, mPAP of (mean ± standard deviation) 50 ± 15 mmHg, and PVR of 10 ± 6 Wood units (Table 1). The majority of the cohort was composed of subjects with either idiopathic PAH (IPAH) (n=870) or CTD-PAH (n=623). The median time from PAH diagnosis to cohort enrollment was 48 months (interquartile range [IQR] 14-92 months). For this analysis,

time under observation began at cohort enrollment, and the cohort was right-censored in July 2018. A total of 1,984 subjects (98.4%) had follow-up data available to ascertain survival. Subjects without follow-up data were excluded from time-to-event analyses. Among these 1,984 subjects, 338 deaths occurred over the follow-up period. The median time from enrollment to death or censor was 41 months (IQR 28-55 months).

Endostatin Associations with Clinical Phenotypes and Outcomes

ES levels for the overall cohort and IPAH and CTD-PAH subgroups are shown in Table 1. ES levels for all PAH subgroups are shown in Supplemental Table 1. ES was highest in subjects with CTD-PAH and lowest in those with portopulmonary hypertension. ES levels among subjects in both of these subgroups were significantly different from those in the overall cohort.

In the overall cohort, each log higher ES was associated with several important disease severity measures, including a 1.84 mmHg higher right atrial pressure (RAP), 1.99 mmHg mPAP, and 0.98 Wood unit higher PVR. Additionally, each log higher ES was associated with a 5 mL lower stroke volume, 190 ml/mmHg lower pulmonary arterial compliance, and a 54-meter shorter 6MWD (Table 2). In subgroup analyses, ES associations with RAP, mPAP, PVR, and 6MWD were of greater magnitude in IPAH compared to CTD-PAH (Supplemental Table 2). Cardiac output (CO) demonstrated a significant association with ES in the IPAH subgroup.

In unadjusted survival analysis, subjects with ES above the cohort median experienced worse survival than subjects with lower ES levels. The association between high ES levels and mortality was also present in unadjusted survival analyses in IPAH and CTD-PAH subgroups (Figure 1, panels A-C). In multivariable Cox proportional hazard models adjusted for multiple

potential confounders of the relationship between serum ES and survival, each log higher ES was associated with a roughly two-fold increased risk of mortality (hazard ratio [HR] 2.32, 95% confidence interval [CI] 1.56-3.45, $p<0.001$) (Table 3). In the IPAH subgroup, each log higher ES was associated with a nearly six-fold increase in mortality (HR 5.68, 95% CI 2.4-12.8, $p<0.001$). In the CTD-PAH subgroup, the magnitude of the relationship between serum ES and mortality was attenuated, and its significance was lost (1.76, 95% CI 0.94-3.32, $p=0.08$).

Incorporating endostatin into ESC/ERS criteria-based risk prediction strategies

Eight hundred and twenty subjects had complete data available to tabulate ESC/ERS low risk criteria. Low-risk criteria include FC I-II, 6MWD >440m, RAP<8mmHg, and CI ≥ 2.5 L/min/m^2 (17, 18). Adding an additional low-risk variable for ES less than the median (37515 pg/mL) improved discrimination of risk groups and produced additional mortality risk strata, as shown in Figures 2a and 2b. Mortality differences between subjects with versus without low-risk ES levels and possessing all four low-risk features are shown in Supplemental Figure 1a. Differences between subjects with versus without low-risk ES levels and with no low-risk features are shown in Supplemental Figure 1b. Univariable and multivariable hazard ratios for each of the low-risk criteria as well as the biomarkers NTproBNP and ES are shown in Table 4. FC, 6MWD, and both biomarkers were significantly predictive of mortality in univariable analysis, though only FC and the two biomarkers NTproBNP and ES remained independently predictive after adjustment for all other low-risk parameters.

A multivariable time-to-event model comprised of the 4 ESC/ERS low-risk categorical variables was significantly improved by adding a variable for ES dichotomized at the median (likelihood ratio [LR] test chi^2 33.78, p<0.001) (Supplemental Table 3). The French Pulmonary Hypertension Registry also assessed a simplified risk scheme composed of three noninvasive variables (FC I-II, 6MWD >440m and NTproBNP <300ng/mL) and found that this simplified scheme also clearly discriminated mortality risk groups among subjects at follow up (17). In our cohort, adding a variable for ES dichotomized at the median to the three noninvasive ESC/ERS variables again improved discrimination of risk groups (Fig 2c and 2d) and model fit (LR chi^2 19.73, p<0.001) (Supplemental Table 3).

To assess the relative utility of models based on invasive versus noninvasive ESC/ERS risk parameters versus models based on biomarkers alone in our cohort, a variety of ESC/ERS criteria-based time-to-event models were constructed and compared using AICs and Harrell's c-statistics in an exploratory analysis. AICs are a means of assessing the relative quality of predictive models by balancing goodness of model fit with model parsimony. The preferred model minimizes AIC, that is, it provides the best possible model fit (e.g., the least information loss about the underlying data) with the fewest possible model terms. Harrell's c-statistic assesses model ability to discriminate censored outcomes such as survival times, with a c-statistic of 0.5 representing a completely uninformative model, and a c-statistic of 1.0 representing a perfectly informative model. In the overall cohort, a biomarker-only model composed of NTproBNP and ES dichotomized at their respective medians outperformed other models by both means of assessment (lowest AIC 1683, C-statistic 0.73), including the full low

risk criteria model, the noninvasive low risk criteria model, and a low-risk criteria model with both NTproBNP and ES added (Supplemental Table 4).

Incorporating endostatin into REVEAL-based risk prediction strategies

One thousand nine hundred and eighty-four subjects had sufficient data to tabulate REVEAL 2.0 risk scores. When REVEAL 1.0 variable cut points were revised for REVEAL 2.0, two new variables (hospitalization within 6 months and estimated glomerular filtration rate) were added to the calculator (15). These new variables are not available in our dataset, however REVEAL was designed for practical use with the clinical data available at any given point in time and has been shown to maintain its predictive power and calibration when at least seven evaluable parameters are available (15, 19). Therefore, REVEAL risk scores (RRS) were tabulated for each subject as long as at least seven evaluable parameters were available for a given subject. Methods for tabulating RRS and dividing risk scores into previously defined risk categories have been previously published (5, 20, 21). The mean ± standard deviation RRS for this cohort was 7.31 ± 2.39, and survival curves for each REVEAL risk category are shown in Figure 3a. Modification of the RRS by subtracting a point for ES below the median and adding a point for ES above the median improved separation among lower risk categories in the first 12-24 months of person-time under observation (Figure 3b).

Univariable and multivariable hazard ratios for each of the categorical REVEAL 2.0 risk parameters as well as ES are shown in Table 5. The majority of REVEAL variables were predictive of mortality in univariable analysis, however in multivariable analysis, only NTproBNP>1100 pg/mL and ES above the median remained independently predictive after

adjustment for all other REVEAL parameters. Among subjects with complete data available for every one of the categorical REVEAL 2.0 parameters available in the cohort (n=438), adding a variable for ES dichotomized at the median improved model fit (LR chi^2 10.51, $p<0.001$) (Supplemental Table 5). Sensitivity analyses were performed to systematically exclude variables with significant missingness, and for each model comparison, addition of ES significantly improved model fit (Supplemental Table 5).

For symmetry with the exploratory analysis conducted to assess the relative utility of various ESC/ERS-based models, a variety of REVEAL-based time-to-event models were constructed and compared using AICs and Harrell's c-statistics (Supplemental Table 6). Models based on categorical REVEAL variables, tabulated RRS, REVEAL risk categories, and biomarkers alone were tested among subjects with complete data available for REVEAL 2.0 parameters. A model including NTproBNP (parameterized per REVEAL 2.0 cutpoints) and ES greater than the median was the best fit, most parsimonious model. A model that added a variable for ES greater than the median to REVEAL 2.0 categorical parameters was the most discriminatory, with C-statistic 0.79.

Discussion

To our knowledge, this is the largest study to examine the angiostatic protein ES as a biomarker of disease severity and survival in PAH. Our findings demonstrate significant associations between circulating ES levels and important measures of disease severity, including hemodynamics and 6MWD. More importantly, our results show strong, significant

associations between ES levels and mortality, particularly in IPAH, even with adjustment for potential confounders and other disease severity markers. Collectively, these results show that ES has clear potential for clinical use as a robust prognostic biomarker in PAH.

Adding information about ES measurements to established PAH risk prediction models improved mortality risk stratification and discrimination. Both ESC/ERS criteria-based and REVEAL 2.0 parameter-based models performed well in our cohort, however we showed that adding ES variables to both risk prediction strategies improved predictive capacity. Furthermore, our analyses demonstrated that unlike many other parameters, biomarker measurements remained strong, independent predictors of survival after adjustment for all other variables included in either risk prediction strategy. Noninvasive and biomarker-only models outperformed (in the case of ESC/ERS-based models) or were similarly informative to (in the case of REVEAL-based models) risk prediction models requiring many more variables, many of which require challenging or invasive means to obtain. Taken together, these results suggest that novel markers like ES could form the cornerstone of future refinements to PAH risk assessment strategies based on noninvasive parameters only.

Our results build upon previous work by our group that indicated ES triggers an angiostatic signal cascade propagated by thrombospondin-1 (TSP-1), inhibitor of differentiation/DNA binding-1 (ID1), and bone morphogenetic protein receptor-2 (BMPR2), which have been directly implicated in the pathogenesis of PAH, to inhibit pulmonary endothelial cell migration, proliferation, and cell survival (11, 22). In addition to effects on the pulmonary vasculature, there is mounting evidence to support a critical role for angiostasis in RV dysfunction and the transition to decompensated RV failure. In a monocrotaline rat model

of PAH, the transition to RV failure is marked by a decrease in angiogenic factors and diminished angiogenesis (12). In humans, decompensated RV failure is associated with greater impairment in angiogenesis than is adaptive RV remodeling (23). Cardiac MRI has shown that clinical RV dysfunction is associated with reduced RV myocardial perfusion reserve in PAH, further suggesting that a failure of angiogenesis to maintain proper myocyte-microvascular balance contributes to RV failure (13). Furthermore, reduced myocardial perfusion reserve is associated with increased mortality in PAH (24). Elevated ES has been observed in the remodeled pulmonary vessels in PAH (25), and studies in coronary artery disease have shown levels of ES in the serum and pericardium correlate with impaired coronary collateral formation (9, 10, 26). Importantly, no studies have directly implicated ES as a regulator of pulmonary vascular or myocardial angiogenesis, and further basic studies are needed to parse the potential effects of ES in pulmonary vasculature versus RV myocardium. However, should ES prove a direct contributor to either pulmonary vascular or RV pathophysiology, this could lend ES a degree of disease specificity not captured by NTproBNP, which reflects nonspecific myocyte stretch.

This study has some limitations. The PAH Biobank does not capture imaging data, and therefore RV metrics are not available to directly examine ES associations with RV function. Our dataset did not capture two newer variables incorporated into REVEAL 2.0, and some REVEAL parameters have missing data, however REVEAL was intended to accommodate missing data, and our results were consistent across several sensitivity analyses excluding variables with significant missingness. While REVEAL and ESC/ERS risk strategies have been tested across multiple cohorts, risk prediction models incorporating ES will require external validation. Our

analyses do not clarify why ES exhibits superior performance characteristics in IPAH versus CTD-PAH. There may be unknown contributors to shortened survival times in CTD-PAH that are not represented in our dataset or accounted for in our models, resulting in residual confounding, or this may simply reflect intrinsic differences in biology between the two predominant PAH subgroups. Finally, we do not have data on subject comorbidities, which may impact survival times, in order to adjust for this important variable in our models.

This study has several strengths. This is the largest study to examine an angiostatic factor as a biomarker in PAH, and our results emphasize the importance of including markers representative of biologically relevant pathways in PAH risk assessment. This is a large, well-phenotyped cohort drawing from multiple pulmonary hypertension referral centers. The large size of the PAH Biobank and the detailed clinical data captured allows for multivariable modeling to adjust for key confounders. Similarly, the large sample sizes available for the two predominant PAH subtypes allow for meaningful subgroup analyses.

In conclusion, ES is a robust prognostic biomarker that is independently predictive of mortality in PAH and may reflect important aspects of disease pathobiology. ES enhances mortality risk prediction when incorporated into existing risk prediction strategies. This study demonstrates clear potential for ES to be incorporated into updated risk stratification tools for PAH. Future studies are needed to determine the potential for ES to serve as a diagnostic or serial biomarker, and to clarify genetic, molecular, and cellular mechanisms. Should future mechanistic studies implicate ES in the causal pathway for PAH development or progression, ES may be a plausible target for drug development efforts.

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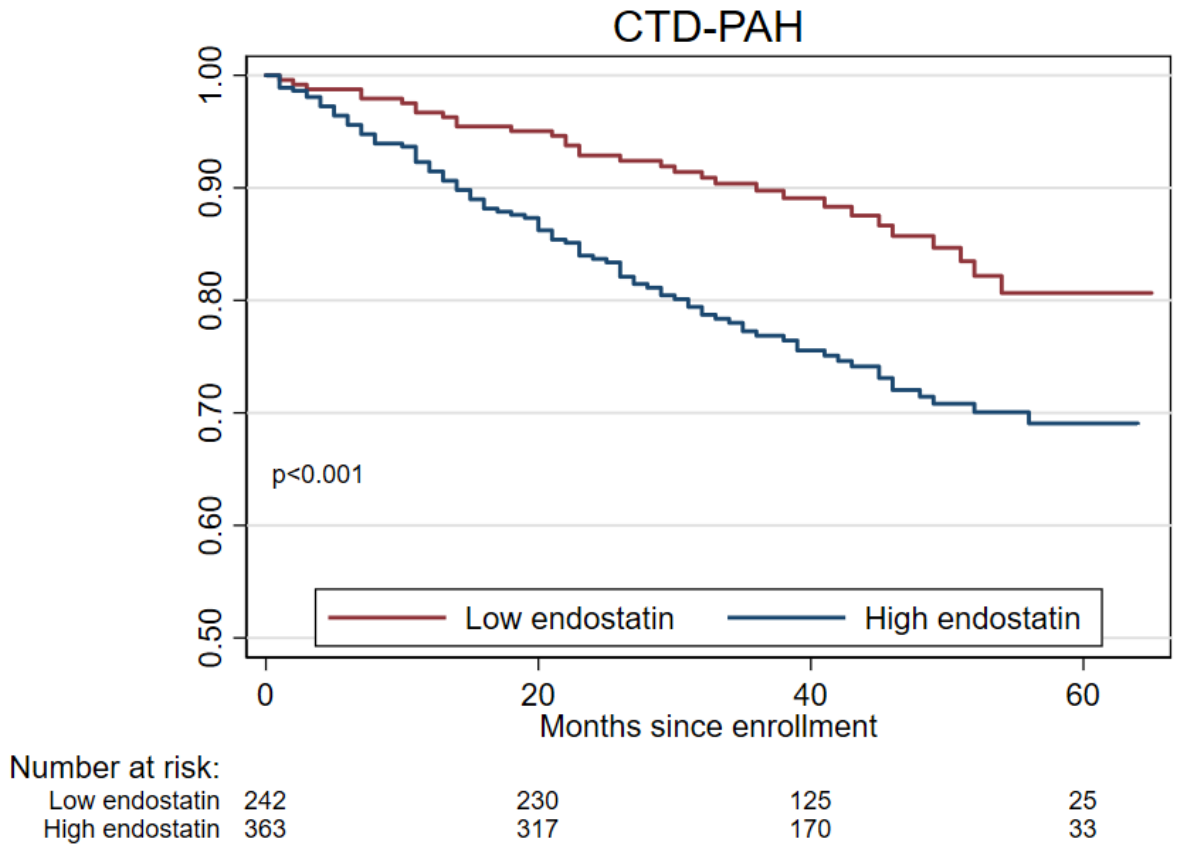
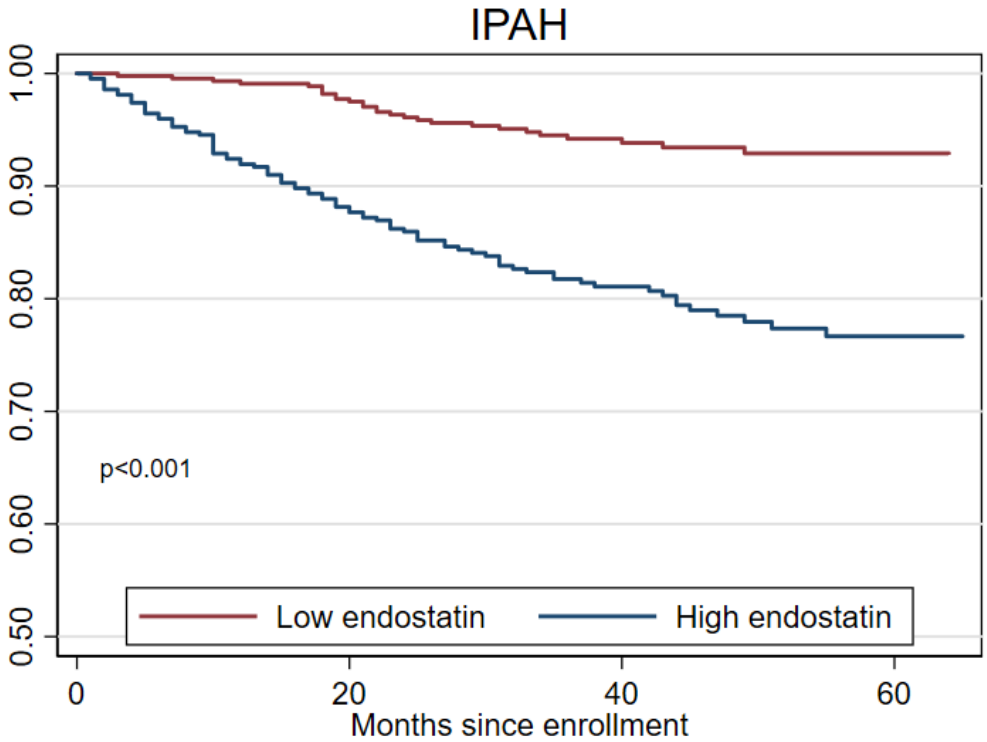
Figure Legends



Number at risk:

Low endostatin	242	230	125	25
High endostatin	363	317	170	33



Number at risk:

Low endostatin	440	430	258	55
High endostatin	422	372	222	50

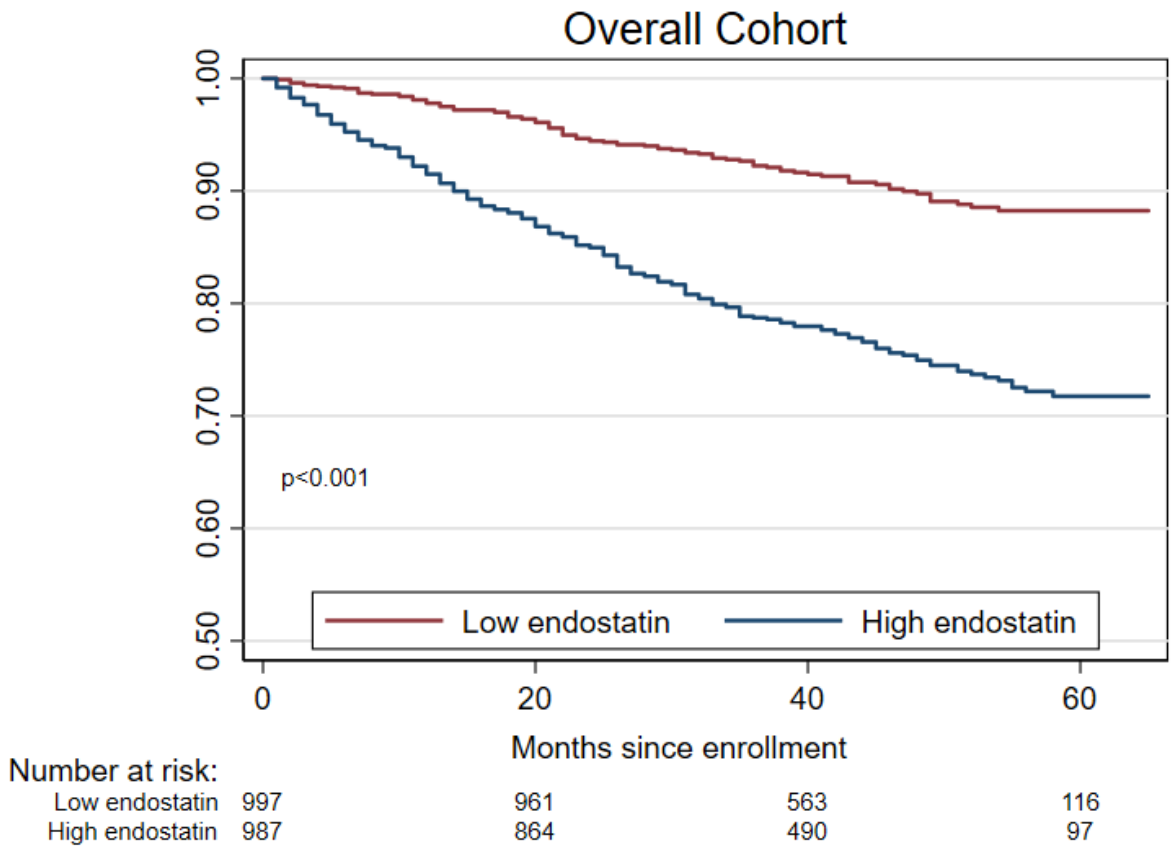
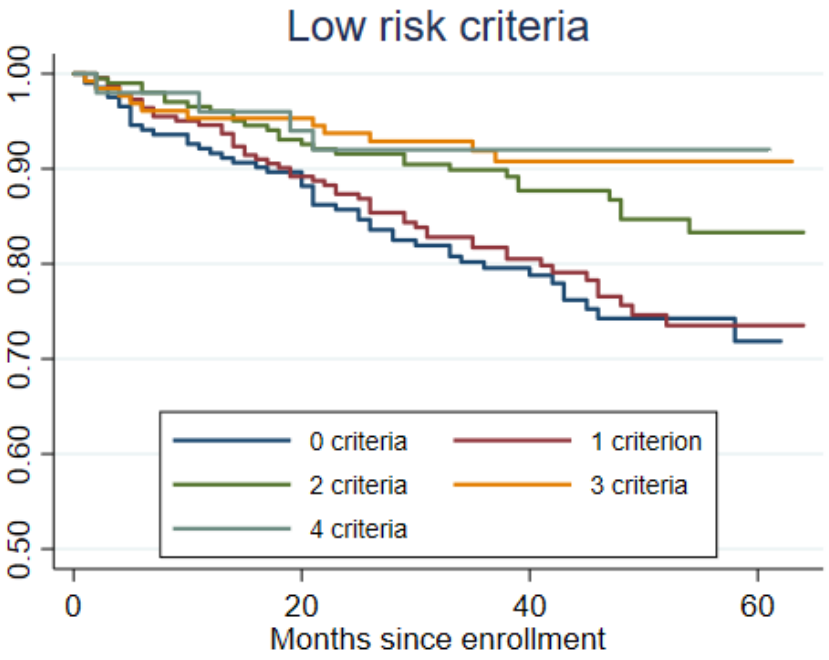
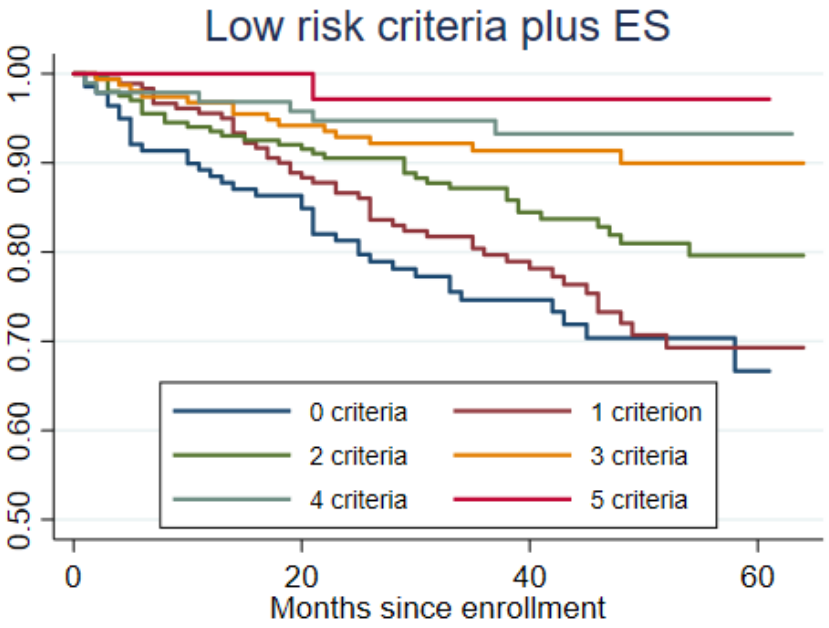


Figure 1. Kaplan-Meier curves depicting associations between ES above vs. below the median and survival in A) the overall cohort, B) IPAH, and C) CTD-PAH.



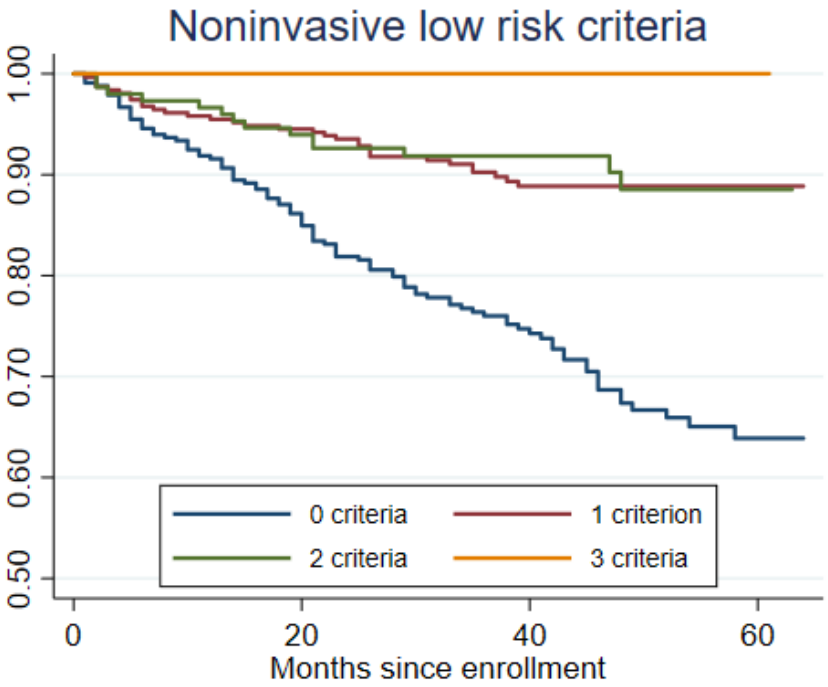
Number of low risk criteria:

0	203	182	105	13
1	222	198	124	26
2	202	188	115	18
3	128	122	68	13
4	50	47	31	4



Number of low risk criteria:

0	139	120	63	9
1	180	160	100	21
2	201	185	119	14
3	155	146	86	19
4	95	91	52	9
5	35	35	23	2



Number of low risk criteria:

0	332	286	161	30
1	310	293	182	28
2	149	140	85	13
3	57	57	35	4

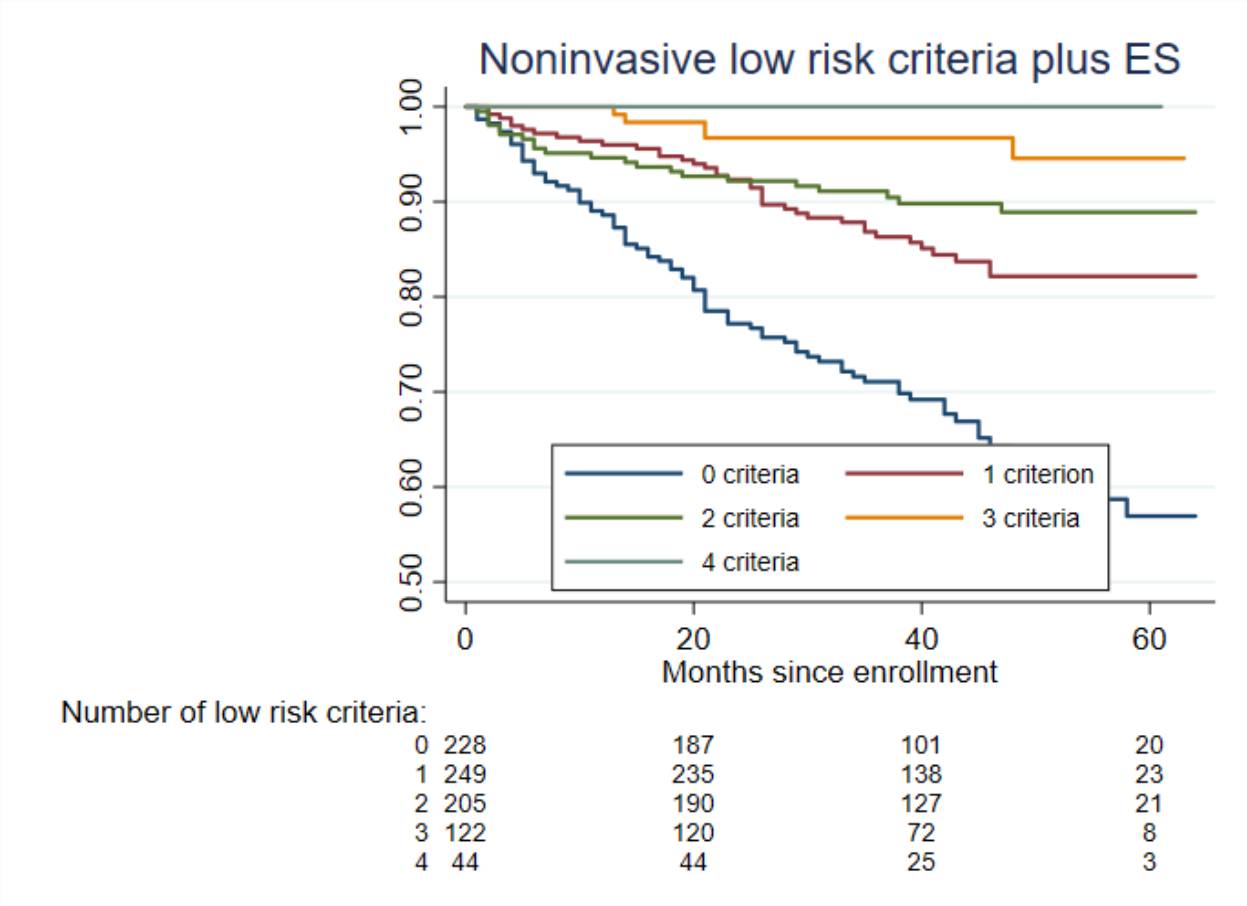
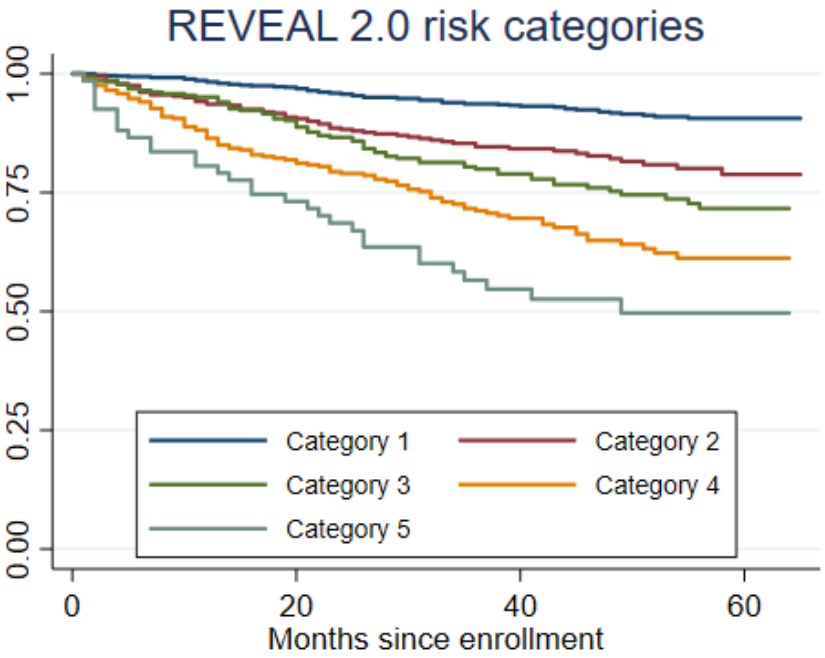


Figure 2. Kaplan-Meier curves depicting survival times by a) number of ESC/ERS low risk criteria applied to the overall cohort, b) number of low risk criteria in the overall cohort after adding an additional low risk variable for endostatin less than the cohort median, c) number of noninvasive low risk criteria in the overall cohort, and d) number of noninvasive low risk criteria in the overall cohort after adding an additional low risk variable for endostatin less than the cohort median.



REVEAL 2.0 risk category:

1	985	957	561	116
2	360	327	199	36
3	285	257	146	33
4	287	235	121	23
5	67	49	26	5

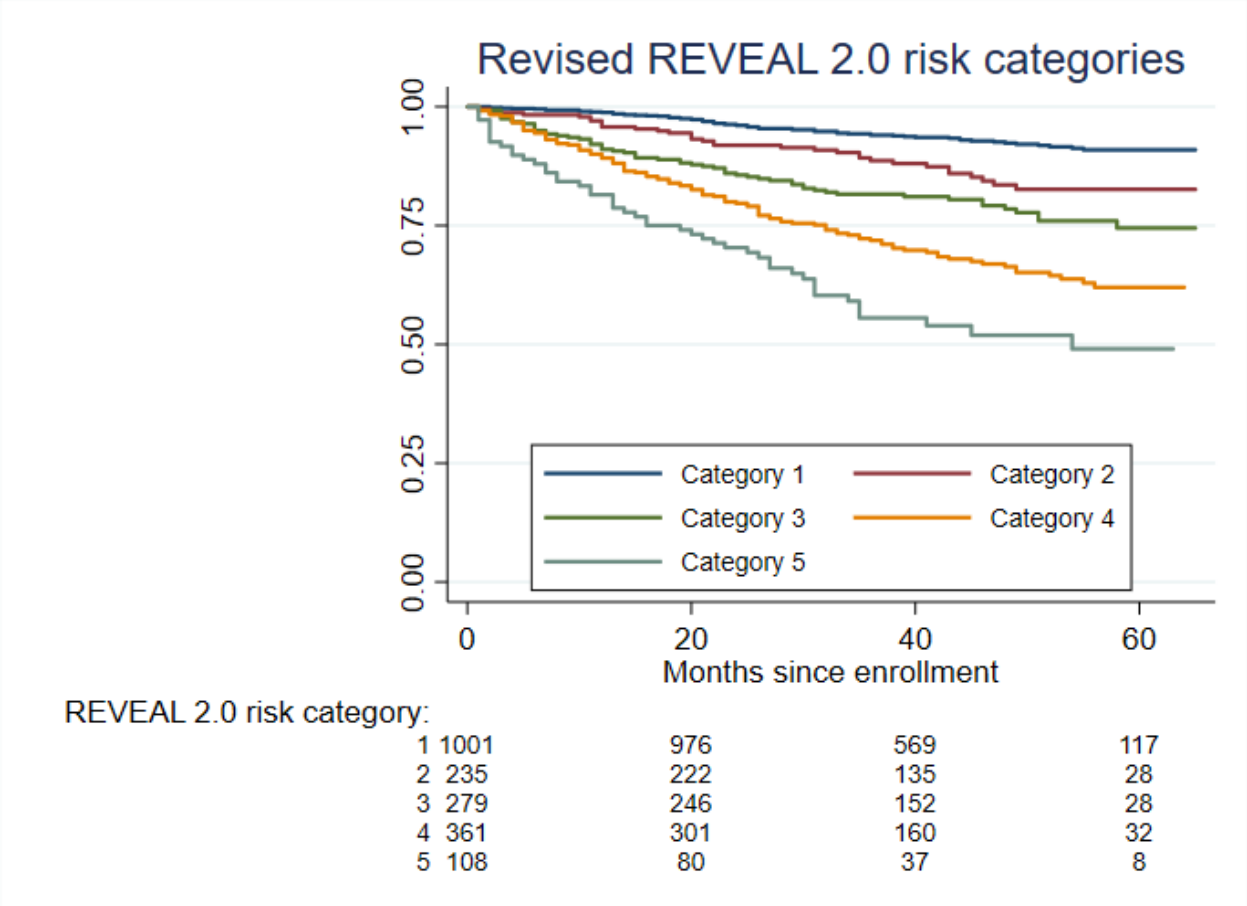


Figure 3. Kaplan-Meier curves depicting survival times by REVEAL 2.0 risk categories based on a) unmodified tabulation of RRS, and b) tabulation of RRS modified by adding -1 for ES below median and +1 for ES above median.

Table 1. Demographics and Clinical Characteristics of the PAH Biobank Cohort

Demographics	**Overall (n=2017)**	**CTD-PAH (n=623)**	**IPAH (n=870)**
Age, years	55 (15)	59 (14)	55 (15)
Sex, n female (%)	1611 (80)	565 (91)	698 (80)
Genetic ancestry, n EA (%)	1662 (82)	564 (91)	780 (90)
Etiology, n FPAH/PVOD/ PortoPulm/ Congenital/Drug/HIV/Other	81/8/111/171 /93/42/18	--	--
NYHA FC, n I/II/III/IV (%III/IV)	90/451/789/118 (45)	24/140/266/34 (65)	38/188/340/56 (64)
6MWD, m	347 (141)	327 (160)	351 (136)
Deaths, n (%)	324 (16)	125 (20)	112 (13)
Biomarkers			
Endostatin, pg/mL (median, IQR)	37,515 (27,946 -50,901)	41,504 (31,639-55,487)	37,087 (27,856-50,028)
NT-proBNP, pg/mL (median, IQR)	672 (217-2,164)	907 (331-3077)	520 (183-1621)
Hemodynamics			
RAP, mmHg	9 (5)	9 (5)	9 (6)
mPAP, mmHg	50 (15)	44 (11)	51 (14)
PAWP, mmHg	10 (4)	10 (4)	10 (4)
PVR, Wood units	10 (6)	8 (5)	10 (6)
Cardiac output, L/min	4.7 (1.7)	4.7 (1.6)	4.6 (1.6)
Cardiac index, L/min/m^2	2.7 (1.2)	2.8 (0.9)	2.6 (1.1)
Therapies, n (%)			
PDE5 inhibitor	1546 (77)	470 (75)	641 (74)
ERA	1205 (60)	370 (59)	515 (59)
IV/SC prostacyclin	699 (35)	161 (26)	355 (41)

CCB	199 (10)	51 (8)	99 (11)

Definition of abbreviations. EA: European ancestry; FPAH: familial PAH; PVOD: pulmonary veno-occlusive disease; Portopulm: portopulmonary hypertension; NYHA FC: New York Heart Association Functional Class; 6MWD: six-minute walking distance; RAP: right atrial pressure; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary artery wedge pressure; PVR: pulmonary vascular resistance; PDE5: phosphodiesterase-5; ERA: endothelin receptor antagonist; IV: intravenous; SC: subcutaneous; CCB: calcium channel blocker.
Values are presented as mean (SD) unless otherwise indicated.

Table 2. Age- and Sex-adjusted ES Associations with Clinical Variables in the Overall PAH Biobank Cohort

Variable	Regression Coefficient
RAP, mmHg	1.84 (1.35-2.33, <0.001)
mPAP, mmHg	1.99 (0.75-3.23, 0.002)
PAWP, mmHg	0.15 (-0.23 – 0.53, 0.45)
PVR, Wood units	0.98 (0.44-1.2, <0.001)
Cardiac output, L/min	-0.14 (-0.30 – 0.03, 0.10)
Cardiac index, L/min/m^2	-0.10 (-0.21-0.01, 0.07)
Stroke Volume, L	-0.005 (-0.008- -0.002, 0.003)
Pulmonary arterial compliance, ml/mmHg	-0.19 (-0.31- -0.08, 0.001)
6MWD, m	-53.5 (-70.7 - -36.2, <0.001)
NTproBNP, pg/mL	1.12 (1.00-1.25, <0.001)

Values are presented as beta coefficient (95% confidence interval, p value) per log ES. See Table 1 for abbreviations.

Table 3. Age- and Sex-Adjusted ES Associations with Survival in the PAH Biobank cohort, overall and by disease subtype

	Univariable HR	**Multivariable adjusted HR***
Overall cohort	3.52 (2.75-4.50, <0.001)	2.32 (1.56-3.45, <0.001)
CTD-PAH subgroup	2.91 (1.93-4.38, <0.001)	1.76 (0.94-3.32, 0.080)
IPAH subgroup	6.44 (4.16-9.98, <0.001)	5.68 (2.4-12.8, <0.001)

Values are presented as hazard ratio (95% confidence interval, p value) per log ES. CTD-PAH: connective tissue disease-associated PAH; IPAH: idiopathic PAH. See Table 1 for all other abbreviations.
**Multivariable models are adjusted for age, sex, PAH-specific therapy, NYHA FC, 6MWD, RAP, mPAP, CI, and PVR*

Table 4. Univariable and multivariable hazard ratios for each of the ESC/ERS low-risk parameters and endostatin less than the median

ESC/ERS Low risk criteria	Unadjusted HR (95% CI)	P value	Adjusted* HR (95% CI)	P value
Functional Class I-II	0.51 (0.38 - 0.68)	<0.001	0.62 (0.41 – 0.94)	0.024
6 min walk distance >440m	0.40 (0.24 - 0.66)	<0.001	0.66 (0.36-1.20)	0.174
RAP <8 mmHg	0.81 (0.65 – 1.02)	0.073	0.78 (0.54-1.14)	0.203
CI ≥ 2.5 L/min/m^2	0.97 (0.78 - 1.21)	0.778	1.01 (0.71 – 1.44)	0.955
NTproBNP <300	0.23 (0.16 – 0.33)	<0.001	0.27 (0.15-0.47)	<0.001
Endostatin < median	0.36 (0.28 – 0.46)	<0.001	0.42 (0.28-0.64)	<0.001

*adjusted for all other variables including biomarkers (NTproBNP <300pg/mL and ES < median)

Table 5. Univariable and multivariable hazard ratios for each of the categorical REVEAL 2.0 risk parameters and endostatin greater than the median

REVEAL parameters	Unadjusted HR (95% CI)	P value	Adjusted* HR (95% CI)	P value
CTD-PAH	1.51 (1.21-1.89)	<0.001	0.90 (0.57-1.44)	0.673
Portopulmonary hypertension	2.19 (1.54-3.11)	<0.001	1.84 (0.81-4.21)	0.146
Heritable PAH	0.65 (0.34-1.27)	0.207	1.44 (0.33-6.21)	0.627
Male > 60 years	1.94 (1.40-2.69)	<0.001	0.79 (0.37-1.71)	0.554
NYHA/WHO FC I	0.59 (0.25-1.37)	0.222	0.27 (0.04-2.08)	0.210
FC II	ref		ref	
FC III	1.82 (1.34-2.47)	<0.001	1.00 (0.55-1.82)	0.997
FC IV	1.92 (1.20-3.09)	0.007	0.97 (0.41-2.30)	0.951
SBP <110 mmHg	0.71 (0.48-1.03)	0.069	0.81 (0.42-1.55)	0.518
HR >96 beats per minute	0.77 (0.50-1.20)	0.255	0.63 (0.29-1.34)	0.231
6MWD <165m	1.75 (1.12-2.71)	0.013	1.28 (0.71-2.32)	0.415
165- <320m	ref		ref	
320- <440m	0.56 (0.39-0.80)	0.002	0.69 (0.41-1.18)	0.178
≥440m	0.35 (0.21-0.59)	<0.001	0.45 (0.18-1.11)	0.082
NTproBNP <300pg/mL	0.49 (0.32-0.74)	0.001	0.52 (0.21-1.25)	0.145
300- <1100pg/mL	ref		ref	
≥1100pg/mL	3.10 (2.35-4.08)	<0.001	3.16 (1.70-5.89)	<0.001
RAP > 20mmHg	1.93 (1.23-3.04)	0.004	0.93 (0.40-2.12)	0.861
PVR < 5 Wood units	1.08 (0.82-1.43)	0.57	1.15 (0.64-2.04)	0.641
Endostatin <median	ref		ref	
>median	2.76 (2.17-3.51)	<0.001	2.47 (1.39-4.39)	0.002

*adjusted for all other REVEAL variables plus endostatin

Online Data Supplement

The angiostatic peptide endostatin enhances mortality risk prediction in PAH

Catherine E. Simpson, MD MHS, Megan Griffiths, MD, Jun Yang, PhD, Melanie K. Nies, MD, R. Dhananjay Vaidya, PhD, Stephanie Brandal, MS, Lisa J. Martin, PhD, Michael W. Pauciulo, MBA, Eric D. Austin, MD MSCI, D. Dunbar Ivy, MD, William C. Nichols, PhD, Allen D. Everett, MD, Paul M. Hassoun, MD, and Rachel L. Damico, MD PhD

Supplemental Figure Legends

Supplemental Figure 1a. Kaplan-Meier plot representing mortality differences in subjects with versus without low-risk endostatin levels AND all four ESC/ERS low-risk features.

Supplemental Figure 1b. Kaplan-Meier plot representing mortality differences in subjects with versus without low-risk endostatin levels with none of the four ESC/ERS low-risk features.

Supplemental Table 1. Endostatin levels by PAH subgroup

PAH subgroup	Endostatin level	p-value
CTD-PAH	41,504 (31,639-55,487)	<0.01
IPAH	37,087 (27,856-50,028)	0.40
FPAH	32,574 (27,395-44,020)	0.03
PVOD	32,566 (24428-42336)	0.30
Portopulmonary Hypertension	30,357 (19,332-47,385)	<0.01
CHD-APAH	32,951 (23,717-43,830)	0.40
Drug/Toxin-Associated PAH	39,259 (28,520-51,322)	0.63
HIV-APAH	32,894 (23,110-44,520)	0.04
Other	36,014 (22,279-45,419)	0.39

Definition of abbreviations: CTD-PAH: connective tissue-associated PAH; IPAH: idiopathic PAH; FPAH: familial PAH; PVOD: pulmonary veno-occlusive disease; CHD-APAH: congenital heart disease-associated PAH; HIV-APAH: HIV-associated PAH.
Values are presented as median (interquartile range).
p-values reflect rank-sum differences between subjects in each indicated subgroup versus all others.

Supplemental Table 2. Age- and Sex-adjusted ES Associations with Clinical Variables in the PAH Biobank Cohort, by disease subtype

Variable	CTD-PAH	IPAH
RAP, mmHg	1.51 (0.65-2.34, 0.001)	2.58 (1.74-3.41, <0.001)
mPAP, mmHg	2.97 (1.13-4.80, 0.002)	3.73 (1.70-5.75, <0.001)
PAWP, mmHg	-0.649 (-1.33-0.03, 0.06)	0.64 (0.02-1.26, 0.04)
PVR, Wood units	1.45 (0.65-2.26, <0.001)	1.54 (0.65-2.42, 0.001)
Cardiac output, L/min	-0.03 (-0.29-0.24, 0.85)	-0.30 (-0.55- -0.04, 0.023)
Cardiac index, L/min/m^2	-0.08 (-0.23-0.06, 0.27)	-0.15 (-0.33- 0.02, 0.09)
Stroke Volume, L	-0.001 (-0.006- 0.003, 0.57)	-0.006 (-0.011- -0.002, 0.009)
Pulmonary arterial compliance, ml/mmHg	-0.24 (-0.47- -0.01, 0.042)	-0.23 (-0.41- -0.06, 0.008)
6MWD, m	-38.8 (-74.8- -28.0, 0.03)	-68.5 (-95.1- -41.9, <0.001)

Values are presented as beta coefficient (95% confidence interval, p value) per log ES. CTD-PAH: connective tissue disease-associated PAH; IPAH: idiopathic PAH. See Table 1 for all other abbreviations.

Supplemental Table 3. Statistical Testing of Extended ESC/ERS Survival Models with Endostatin Added Compared to Null Models without Endostatin with Likelihood Ratio Tests

ESC/ERS Null Models	Extended Models	LR X^2	*p* value
4 low risk criteria*	4 low risk criteria + ES***	33.78	<0.001
Noninvasive low risk criteria**	Noninvasive low risk criteria + ES	19.73	<0.001

Definition of abbreviations. LR $X^{2:}$ likelihood ratio chi-squared.
*Low risk criteria are FC I-II, 6MWD >440m, RAP<8mmHg, and CI ≥ 2.5 L/min/m2
**Noninvasive criteria are FC I-II, 6MWD >440m, NTproBNP <300pg/mL
***ES < median 37515 pg/mL

Supplemental Table 4. Statistical Testing of ESC/ERS-Based Survival Models with AICs, BICs, and C-statistics

ESC/ERS-based model variables	Degrees of freedom	AIC	BIC	C-statistic
4 low risk criteria*	4	1763.436	1782.200	0.6166
4 low risk criteria + ES	5	1731.659	1755.113	0.6848
4 low risk criteria + NTproBNP	5	1722.46	1745.914	0.6904
4 low risk criteria + ES + NTproBNP	6	1705.497	1733.642	0.7183
Noninvasive low risk criteria**	3	1721.489	1735.562	0.6791
Noninvasive low risk criteria + ES	3	1703.224	1721.988	0.7118
NTproBNP <300pg/mL	1	1736.895	1741.586	0.6253
ES < median***	1	1737.830	1742.521	0.6445
NTproBNP <300pg/mL + ES < median	2	1710.770	1720.151	0.6949
NTproBNP < median****	1	1699.755	1704.446	0.6876
NTproBNP <median + ES < median	2	**1683.201**	**1692.582**	**0.7283**

Definition of abbreviations. AIC: Akaike information criteria; BIC: Bayesian information criteria.
*Low risk criteria are FC I-II, 6MWD >440m, RAP<8mmHg, and CI ≥ 2.5 L/min/m2
**Noninvasive low risk criteria are FC I-II, 6MWD >440m, NTproBNP <300 pg/mL
***ES median 37514.91 pg/mL
**** NTproBNP median 672.559 pg/mL
Lowest AIC/BIC and highest C-statistic are bolded

Supplemental Table 5. Statistical Testing of Extended REVEAL 2.0 Models with Endostatin Added Compared to Null Models without Endostatin with Likelihood Ratio Tests

REVEAL Null Models	Extended Models	LR X^2	*p* value	N
REVEAL parameters	REVEAL parameters + ES>median	10.51	0.001	438
Sensitivity analyses:				
REVEAL parameters, except 6MWD	REVEAL parameters, except 6MWD + ES>median	16.98	<0.001	670
REVEAL parameters, except HR and SBP	REVEAL parameters, except HR and SBP + ES>median	7.51	0.023	787
REVEAL parameters, except NYHA FC	REVEAL parameters, except NYHA FC + ES>median	11.34	0.003	520

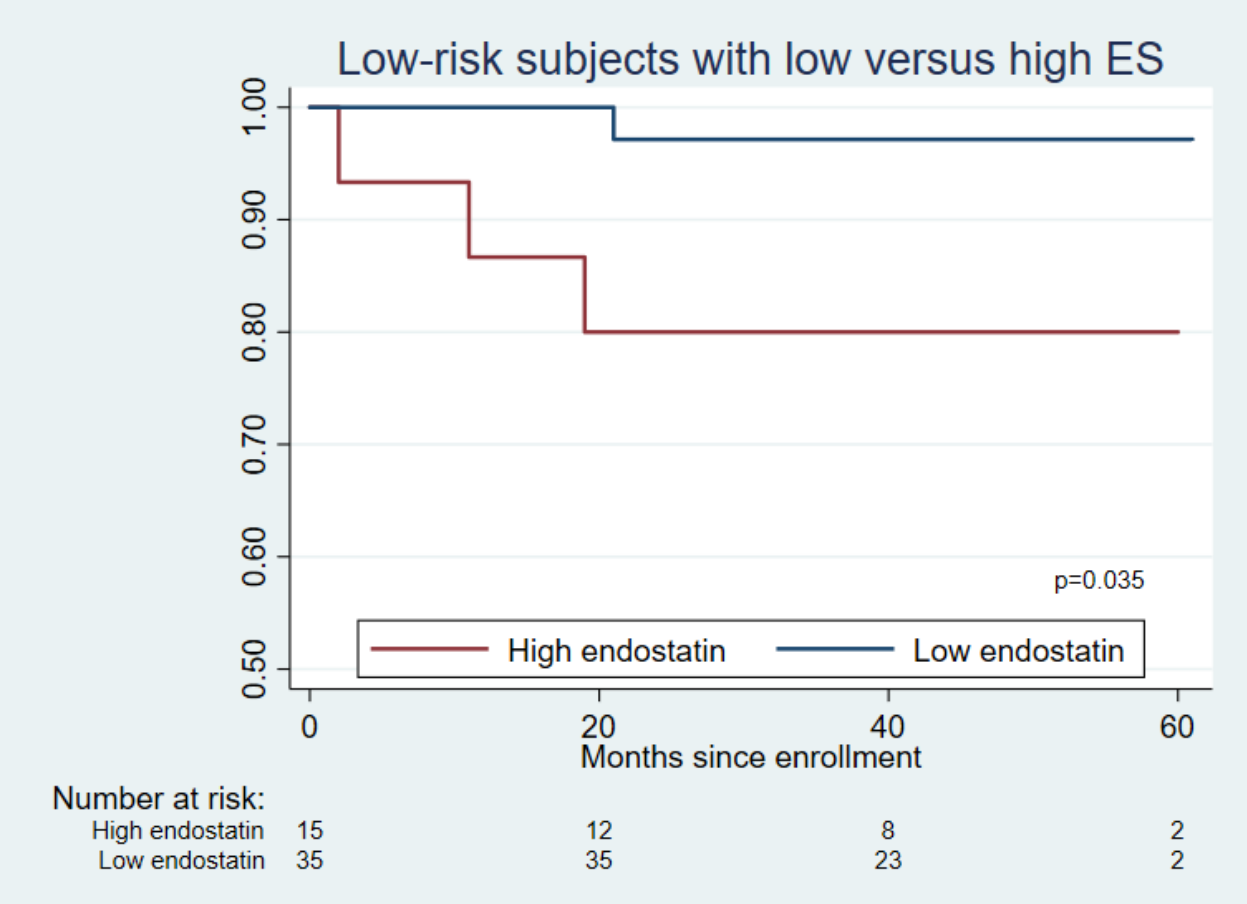
Definition of abbreviations. LR X^2: likelihood ratio chi-squared. See Table 1 for other abbreviations.
REVEAL parameters: presence of CTD-PAH, portopulmonary hypertension, heritable PAH; male > 60 years NYHA/WHO FC I, FC II,FC III,FC IV; SBP <110 mmHg; HR >96 beats per minute; 6MWD <165m, 165- <320m, 320- <440m, ≥440m; NTproBNP <300pg/mL, 300- <1100pg/mL, ≥1100pg/mL; RAP > 20mmHg; PVR < 5 Wood units

Supplemental Table 6. Statistical Testing of REVEAL-Based Survival Models with AICs, BICs and C-statistics for subjects without any missing data for REVEAL parameters (n=438)

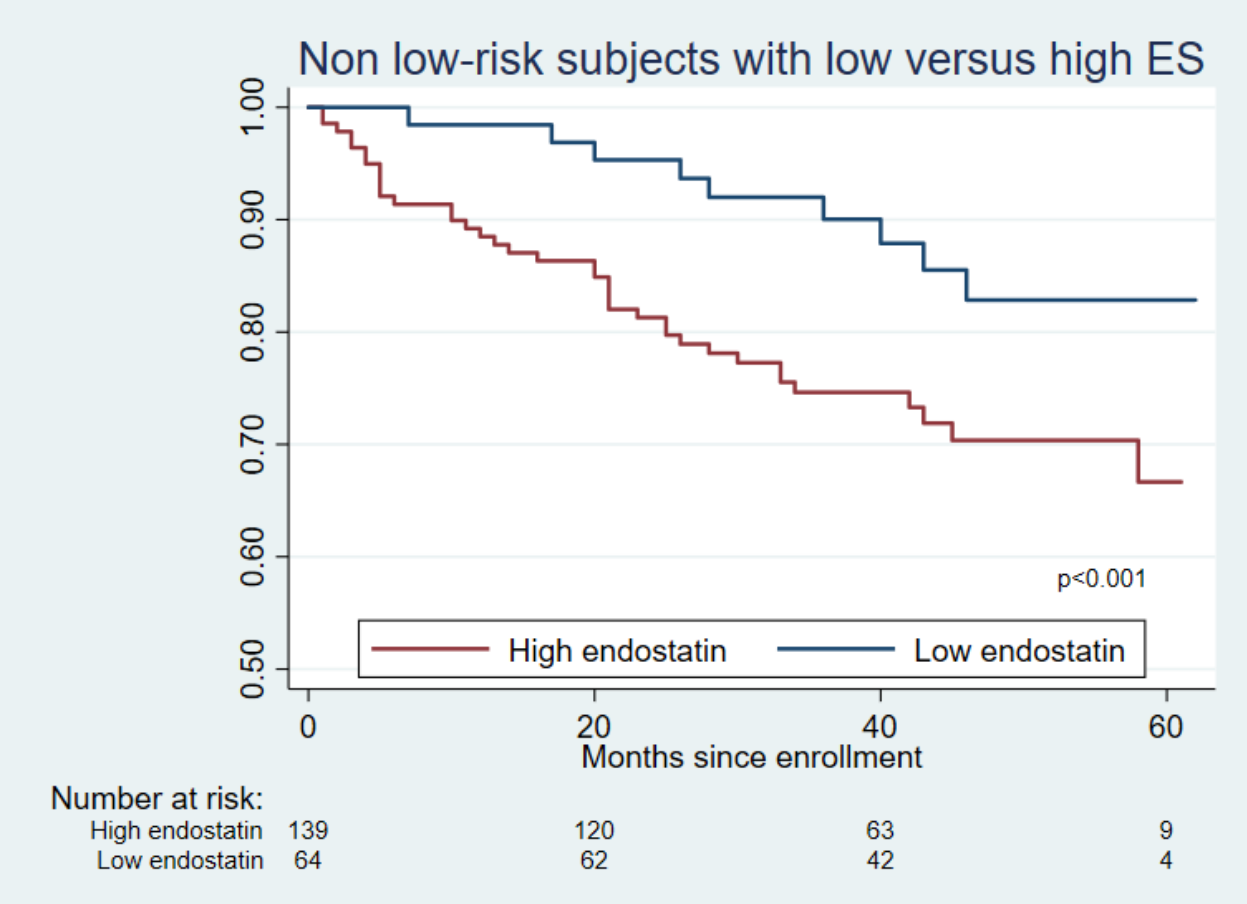
REVEAL-based model variables	Degrees of freedom	AIC	BIC	C-statistic
REVEAL 2.0 parameters	16	966.413	1031.728	0.7828
REVEAL 2.0 parameters + ES>median	17	957.8997	1027.297	**0.7932**
REVEAL 2.0 risk scores	11	994.5804	1039.485	0.6999
REVEAL 2.0 risk scores + ES>median	13	982.1773	1035.246	0.7374
REVEAL 2.0 risk categories	4	987.8481	1004.177	0.6831
REVEAL 2.0 risk categories + ES	4	972.0048	988.3337	0.7205
REVEAL 2.0 NTproBNP	2	960.4595	968.6239	0.7256
ES > median	1	988.2197	992.302	0.6653
REVEAL 2.0 NTproBNP + ES > median	3	**946.4291**	**958.6758**	0.7590
NTproBNP > median	1	970.6574	974.7396	0.6949
NTproBNP >median + ES >median	2	953.8023	961.9668	0.7428

REVEAL parameters: presence of CTD-PAH, portopulmonary hypertension, heritable PAH; male > 60 years NYHA/WHO FC I, FC II,FC III,FC IV; SBP <110 mmHg; HR >96 beats per minute; 6MWD <165m, 165- <320m, 320- <440m, ≥440m; NTproBNP <300pg/mL, 300- <1100pg/mL, ≥1100pg/mL; RAP > 20mmHg; PVR < 5 Wood units; REVEAL risk scores range 1-15; REVEAL risk categories range 1-5.

Lowest AIC/BIC and highest C-statistic are bolded



Supplemental Figure 1a



Supplemental Figure 1b.