Official Study Title: Effects of Dehydroepiandrosterone in Pulmonary Hypertension

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Research Strategy

A. Significance

A1. Sexual dimorphism in PH prevalence and outcomes. Female sex is the strongest clinical risk factor for PAH, with a 4:1 female-to-male ratio reported from the largest registry.[31] Despite the increased risk of PAH in women, women with PAH have better survival than men.[1, 32] RV function is an important cause of morbidity and mortality in PAH as well as highly prevalent heart and lung disease but determinants of the RV response are entirely unknown.[7-9] We and others have shown that female sex is associated with better RV systolic function in both health and disease, including PAH and left heart failure.[2, 33-35] Targeted PAH therapy leads to greater improvements in RVEF (demonstrated after just several months of treatment) in women as compared to men and partially explains better outcomes in women.[4, 36] Demonstration that DHEA has direct RV and sex-based effects will support the hypothesis that sex hormones play an important role in disease pathogenesis and provide insight into sex hormone manipulation as a treatment strategy in PAH.

A2. Beneficial effects of DHEA on pulmonary vasculature. DHEA and DHEA-S are precursors in the biosynthesis of androgens and estrogens (Figure 2), but we and others have shown biological effects of DHEA which are independent of other sex hormones. As PAH is more common in women than in men, estrogen has been implicated as a mechanistic factor in disease development and in fact estrogen can induce PH in some animal models.[40] In contrast, DHEA appears to be consistently beneficial in experimental PH via mechanisms which are entirely independent of E2 signaling (Table 1), although these studies have been done predominantly in male animals (as with most published studies in animal models for PH).[16-20, 39] In humans, lower DHEA-S levels have been linked to systemic endothelial dysfunction and track independently with flow mediated dilatation; these effects can be rescued with DHEA supplementation.[41, 42] A recent study of unbiased metabolomic profiling demonstrated DHEA-S and its metabolites were reduced in PAH patients compared to healthy controls; lower circulating levels of DHEA-S were associated with mortality in PAH.[14]

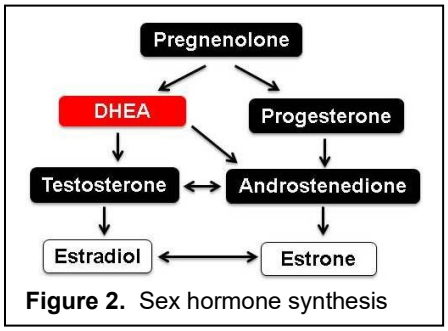


Figure 2. Sex hormone synthesis

Table 1. Experimental data supporting beneficial effects of DHEA

Model	DHEA Effects	Proposed Mechanism
Hypoxia	Prevent, rescue PH	BK_{Ca} channels [16, 17]
	Rescue PH	BK_{Ca}, inhibits 5HT-, KCl-induced SMC growth [37]
	Rescue RV	Reduces cardiomyocyte proliferation[18]
Altitude	Prevent, rescue PH	sGC [38]
MCT, PASMCs	Prevent, rescue PH	Inhibits Src, STAT3, Pim1 Increases BMPR2, miR-204 [20]
MCT-PNX	Prevent, rescue	Inhibits RhoA/Rho kinase [39]
SU5416/ Hypoxia	Rescue RV>PH	Reduces RV capillary rarefaction, apoptosis, ROS via STAT3 [19]
Cardiomyocytes	Antichronotropic, Antihypertrophic	Reduces T-type Ca channels[22] Inhibits natriuretic peptide
	Antihypertrophic	Reduces ET-1 induced hypertrophy[21] Inhibits BNP

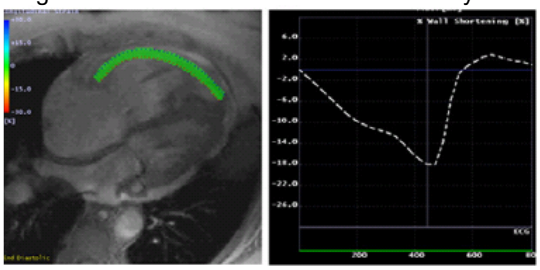
BK_{Ca}=large conductance Ca^{2+}-activated K channel; 5HT=serotonin; SMC=smooth muscle cell; STAT3=signal transducer and activator of transcription 3; BMPR2=bone morphogenetic receptor type II; MCT=monocrotaline; PNX=pneumonectomy

A3. DHEA and cardiac function. Low DHEA-S levels have been associated with an increased risk of death in heart disease, cardiac allograft vasculopathy, and heart failure severity.[43-47] DHEA reverses cardiomyocyte hypertrophy induced by ET-1[21] and prevents myocardial fibrosis and contractile dysfunction by restoring oxidative balance, mitigating the actions of AGE products and its receptors, and improving tissue levels of collagen and fibronectin.[48, 49] Myocardial fibrosis is a key phenotypic change in RV remodeling and decompensation in PAH.[50] DHEA treatment improves cardiac index and inhibits RV capillary rarefaction, fibrosis, and oxidative stress, suggesting an RV-disproportionate or -specific effect.[19] While first established as major regulators of pulmonary vascular tone, NO and ET-1 also influence RV phenotype.[30, 51] There is a DHEA receptor coupled to endothelial NO synthase (eNOS) and DHEA regulates ET-1 synthesis and inhibits ET-1 promoter activity.[23, 24] Our data show a definitive link between lower DHEA-S levels, PAH and RV function in men and women (Section C3), independent of E2. DHEA's key links with RV remodeling, NO and ET-1 could explain sex-based differences in outcomes and PAH therapeutic response (Figure 1). If we see that DHEA effects RV function, we will next validate these findings in a multicenter, parallel arm, Phase II RCT.

A4. Important markers of RV phenotype and oxidative stress. The RV as compared to the left ventricle (LV) has complex rotational motion from a unique myocardial fiber arrangement requiring highly sensitive methods to detect conformational changes. RV metrics obtained by cardiac MRI are highly accurate and MRI is the gold standard for RV assessment.[52-55] **Longitudinal strain**, which is an angle-independent method of assessing RV systolic function measured by echocardiography is associated with survival in PAH and tracks with PAH therapy.[56, 57] Echocardiography is inferior to MRI, however, and pixel based multimodality tissue

tracking (MTT) using MRI cine images is now available to quantify strain. This method has been described in PAH (Figure 3) and is highly reproducible, with intra- and inter-observer mean differences of -0.5 ± 1.5% and -0.6 ± 2.1% respectively.[58] Assessment of strain by MRI appears highly sensitive for the detection of early pre-clinical changes in RV contractility, making this an ideal primary end point to detect an efficacy signal in this "proof of concept" study. **RVEF** is a key determinant of outcome in RV failure regardless of etiology including PAH.[7-9] RVEF predicts outcome in PAH and changes in RV measures track with even short-term (<1 hour, typically several months) exposure to PAH therapies.[4, 36, 59-61]

Figure 3. Representative RV strain analysis, MTT. Green line=RV strain curve. Right box is longitudinal strain values over the cardiac cycle.



Galectin-3, a marker of myocardial extracellular matrix metabolism and fibrosis, is elevated in the plasma of PAH patients[62] and inversely correlates with MRI-based measures including RVEF.[63] **RAGE** levels are elevated in plasma and explanted lungs of PAH patients.[64, 65] RAGE is a strong STAT3 activator (key in PAH pathophysiology and one of the mechanisms by which DHEA reverses experimental PH).[20, 65] Soluble RAGE has been shown to inhibit myocardial apoptosis via STAT3 activation in cardiac ischemia/reperfusion injury.[66] While **oxidative stress** is challenging to quantify in patients, we propose measuring the levels of MPO, a major source of ROS linked to systemic inflammation with a track record in cardiovascular disease as a redox biomarker,[67-69] and sNOX2-dp, a marker of NADPH oxidase activity, which predicts microvascular obstruction in coronary disease.[70-72]

A5. DHEA's efficacy in clinical trials and evidence of safety. Most RCTs of DHEA have been conducted in adrenal insufficiency and systemic lupus erythematosus. No serious side effects were reported in these RCTs including >1200 patients, with reversible mild androgenic side effects (oily skin, hirsutism, acne) in some studies.[73-75] DHEA 50 mg daily improved systemic vascular stiffness and reduced interleukin (IL)-6 as well as tumor necrosis factor-α in healthy adults [76], cytokines implicated in PAH pathogenesis and associated with symptoms and survival in PAH[77, 78]. In eight patients with PH related to COPD, treatment with three months of DHEA was associated with a significant increase in six-minute walk distance (6MWD) and improvements in hemodynamics without adverse effects.[79] While this small study is promising, it was uncontrolled, open-label, and did not include patients with PAH. We have studied anastrozole as an approach to reducing E2 levels in PAH (Section C4). There was no effect on DHEA-S levels[15], so that the observed reductions in E2 and increase in 6MWD were unrelated to DHEA. Furthermore, our preliminary data show that DHEA is linked to the risk of more severe disease independent of changes in E2 and other hormones. Considering the consistently favorable findings in experimental and human PAH and the low risk of the intervention, there is a strong scientific premise and critical need for a clinical trial examining DHEA in the treatment of PAH.

A6. DHEA alters phenotype of cultured endothelial cells. DHEA at variable concentrations (10^{-5}–10^{-8} M) induces changes in human endothelial cell phenotype.[20, 80-84] Effects include enhanced eNOS expression and NO synthesis and modulated ET-1 secretion.[24, 82] DHEA reduces inflammatory signaling implicated in atherosclerosis in human systemic vein endothelial cells.[80, 84] Human PAECs actively metabolize DHEA and treatment of PAECs from PAH patients decreases STAT3 activation,[20, 85] an important mediator of pulmonary vascular remodeling. The effect of DHEA treatment on pulmonary endothelial NO and ET-1 synthesis in PAH has not been studied nor has the effect of patient sex on PAEC response to DHEA treatment.

A7. Intersection of sex and sex hormones with key mechanistic pathways in PAH and the RV. Extensive data from preclinical models have implicated sex hormones in PH but translation of these findings to human PAH has lagged behind. We (and others) have recently shown that men with PAH have worse hemodynamics as compared to women with PAH[5, 86] and that sex hormone levels as well as genetic variants in hormone pathways are linked to PAH risk and RV morphology.[2, 87, 88] Men with PAH are more likely to respond to PDE5i (a major class of PAH treatment), while women with PAH respond better to ERAs (another widely used therapy) than men.[3, 6] Differential responses to therapy may be due to the interaction of key mechanistic pathways in PAH with DHEA (Figure 1). Women have greater levels of NO biosynthesis compared to men, while men have higher levels of ET-1 and greater ET-1-mediated vasconstriction.[25, 27, 29] Sex hormones directly modulate NO/cyclic guanosine monophosphate (cGMP) signaling and circulating ET-1 levels.[28, 89, 90] Animal models of PH have increased female penetrance and show detrimental effects of E2 on the pulmonary vasculature under some but not all conditions.[91-94] Unlike E2, which has conflicting and paradoxical effects on cardiopulmonary function, DHEA shows consistently beneficial effects on the pulmonary vasculature *and* the

right heart, providing a strong scientific premise to proceed with a human pilot trial of DHEA.

A8. Critical need to improve treatment of PAH. PAH treatment is extremely expensive[95, 96] and annual medication costs exceed $200,000. While a recent trial suggests a benefit of up-front combination treatment with PDE5i and ERA[3, 6, 97], it is possible that the combination approach simply assures a greater proportion of individuals receive the most effective drug for their sex or hormonal milieu. Understanding treatment response heterogeneity is critical for cost containment and for the advancement of precision medicine in PAH. If we show sex-based differential responses to DHEA (*in vivo* or in PAECs *in vitro*), this will inform our understanding of why treatment responses with PAH therapies differ by sex and allow for the adaptive design of future RCTs.

A9. Scientific Premise. Preclinical and limited human studies indicate that DHEA favorably impacts endothelial cell-derived vasoactive mediators and myocardial remodeling. We have shown that men and women with PAH have lower levels of DHEA-S and that low levels predict more severe disease independent of E2 levels (Section C3); a recent study linked low DHEA-S levels to mortality in PAH.[14] Therefore, there is a need for determining the safety and efficacy of DHEA in humans with PAH. Only an interventional study with a complementary cell based aim can untangle the role of DHEA in cardiopulmonary function in PAH.

B. Innovation

Conceptual Innovation:

- We will test the idea that DHEA, an endogenous hormone, alters RV and endothelial phenotype in PAH.
- Restoration of low DHEA levels may correct the derangements that lead to PAH and have direct effects on RV adaptation rather than simply combating vasoconstriction as is the case for all currently available pulmonary vasodilators.
- DHEA is an inexpensive and safe supplement not previously studied in human PAH and RV failure that has effects independent of those of other sex hormones, like E2.
- These studies will have implications for precision care in PAH in that already available therapies may be preferentially advised based on an individual's sex or sex hormone milieu. Our objectives for this proposal and beyond are well-aligned with the NIH Strategic Plan for Lung Vascular Research[98] and a recent workshop on enhancing treatments for PAH through precision medicine.[99]

Technical Innovation:

- To our knowledge, there are no RCTs of this size in PAH with the primary end point of MRI-derived RVEF – the proximate cause of death in PAH and PH for which there are no approved therapies.
- The use of a cross-over design with carefully chosen end points to capture RV phenotype (imaging, markers of RV fibrosis and oxidative stress), known major mechanistic mediators in PAH (NO and ET-1), as well as common intermediate end points in PAH (6MWT, health-related quality of life [HRQOL], functional class) will maximize precision, minimize within subject variability, and allow for robust patient-level analyses to detect variable signals based on an individual's sex, age, race/ethnicity, and PAH sub-type.
- Mechanistic studies of DHEA's effects will use PAECs isolated from PAH patients, a very novel approach.

C. Approach

C1. Men have worse disease than women with PAH. First, we determined whether men and women have baseline differences that may explain distinct PAH epidemiology and outcomes. In a large (n = 1211), pooled analysis of patient-level data from Phase III RCTs in PAH submitted to the FDA, men with PAH had higher right atrial pressure (RAP) and pulmonary vascular resistance (PVR) compared to women with PAH (Table 2).[5] Younger men had higher mean pulmonary artery pressures (mPAP) than younger women.

Table 2. Sex related differences in hemodynamic values in 1211 PAH patients

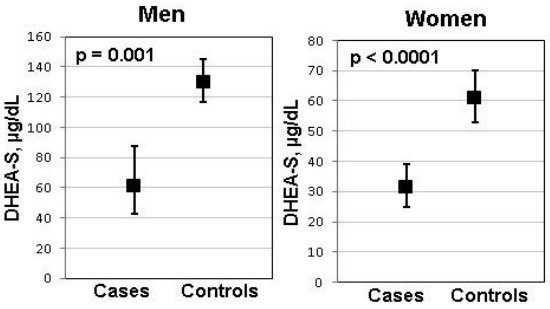
Variable	Age	Difference, men and women	95% CI	P value
RAP, mm Hg	.	1.36	0.44 – 2.27	0.004
mPAP, mm Hg	< 45	5.43	2.20 – 8.66	0.001
	45-54	1.37	-2.64 – 5.37	0.50
	55-64	-1.45	-5.70 – 2.81	0.50
	65+	3.06	-1.31 – 7.43	0.17
Cardiac output, L/min	.	-0.21	-0.43 – 0.01	0.06
PVR, Wood units	.	1.23	0.18 – 2.27	0.02

Adjusted for age (if no sex*age interaction), race, height, weight, study

C2. Sex hormones and RV function. We next sought to determine whether sex hormone levels are associated with RV function as an explanation for dimorphic outcomes in PAH. We examined sex hormone levels from a large (n = 3695) cardiovascular disease-free cohort (Multi-Ethnic Study of Atherosclerosis [MESA]) generalizable to RV failure irrespective of cause.[2, 100] Higher levels of DHEA were associated with lower RVEF (calculated from RV stroke volume/RV end-diastolic volume), higher RV stroke volume (calculated from RV end-diastolic – RV end-systolic volume), and larger RV end-diastolic volume in healthy post-

menopausal women. While a lower RVEF and larger RV stroke volume seem difficult to reconcile, higher (but not necessarily detrimental) RV end-diastolic volume with increasing DHEA levels would result in larger RV stroke volume (which may be beneficial) and numerically but not necessarily pathologically lower RVEF. In fact, virtually all participants had a normal RVEF in MESA as these are "disease-free" adults. Our data in patients with PAH show that higher DHEA-S levels *protect against PAH and improve RV function* (Section C3), apparently contradicting the MESA findings. These inconsistencies likely result from the measurement of DHEA-S (in PAH) versus DHEA (in MESA)(the former having more stability), studying a diseased versus a healthy population, and the analysis of DHEA's cross-sectional associations with normal RV indices in MESA. The only way to answer these questions is an interventional study of DHEA in PAH as proposed.

Figure 4. DHEA-S levels in PAH cases vs. controls adjusted for age and BMI.



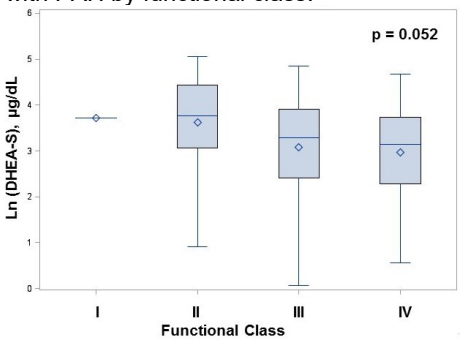
C3. DHEA in human PAH. Given our observations in healthy participants from MESA, we studied whether DHEA and other sex hormones were risk factors for PAH in men[101] and women. We included 23 men with PAH matched by age and body mass index to 67 controls without clinical cardiovascular disease. We recently assessed 112 post-menopausal women with PAH age- and body mass index-matched to 148 controls. DHEA-S levels were significantly (50%) lower in PAH cases vs. controls in both men (p = 0.001) and women (p < 0.0001) (Figure 4). Similar to our published findings in men, every 10 Ln(µg/dL) decrease in DHEA-S levels increased the odds of PAH by 26% (95% confidence interval 13%–39%, p < 0.0001) in women.

Table 3. Lower DHEA-S levels and markers of disease severity in men and women with PAH

	Men			Women		
Variable	**Estimate**	**95% CI**	**P value**	**Estimate**	**95% CI**	**P value**
6MWD, m	-6.1	-47.1, 34.9	0.750	-30.2	-54.0, -6.5	0.013
RAP, mm Hg	2.7	-0.6, 4.8	0.020	1.6	0.3, 2.8	0.019
mPAP, mm Hg	0.0	-5.4, 5.3	0.999	3.6	1.8, 5.4	<0.001
Cardiac output, L/min	-0.6	-1.4, 0.2	0.140	-0.2	-0.4, 0.0	0.067
PVR, Wood units	3.4	0.9, 5.9	0.010	0.9	0.3, 1.5	0.002

Adjusted for age and body mass index

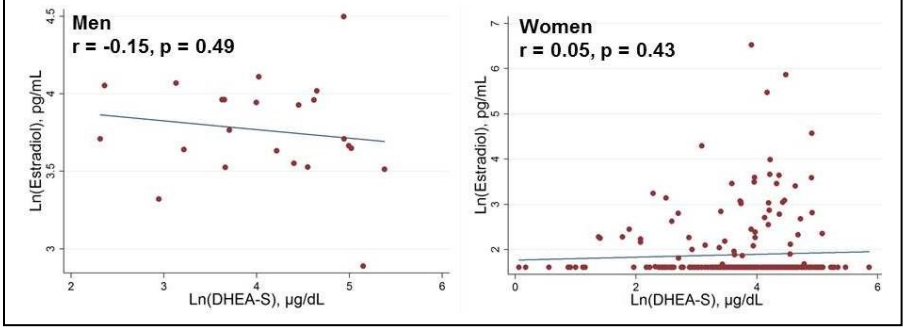
Figure 5. Ln(DHEA-S) levels in women with PAH by functional class.



In both men and women, lower levels of DHEA-S were associated with more severe disease including markers of RV function (Table 3). For example, each 1 Ln(µg/dL) decrease in DHEA-S levels was associated with a 3 mm Hg higher RAP (p = 0.020) and a 3 Wood unit higher PVR (p = 0.010) in men, and a 2 mm Hg higher RAP (p = 0.019) and 1 Wood unit higher PVR (p = 0.002) in women. The relationships between DHEA-S levels and markers of disease severity were more robust in women than in men. In women, each 1 Ln (µg/dL) decrease was associated with a 30 meter shorter 6MWD (p = 0.013) (approaching the minimal clinically important difference in PAH)[102], as well as a 4 mm Hg increase in mPAP (p = <0.001), and may have been associated with a 0.2 L/min decrease in cardiac output (p = 0.067), a trend that was also noted in men. There was also a borderline significant inverse relationship between levels of DHEA-S and functional class in women (Figure 5); patients with lower DHEA-S levels had more symptoms (higher functional class).

It was possible that DHEA-S was exerting effects via E2. However, there was no significant correlation between DHEA-S levels and E2 levels in men (r = -0.15) or women (r = 0.05) with PAH (Figure 6). In addition, the associations between DHEA-S levels and the risk of PAH and all clinical severity measures were unaffected when E2 was added to the models, indicating the demonstrated

Figure 6. Lack of correlation between DHEA-S levels and estradiol levels in men and women with PAH.



relationships were independent of E2 (and other hormones). There were also no significant interactions between DHEA-S levels and E2 levels (and other hormones) and the risk of PAH nor disease severity markers. In other words, E2 levels did not modify (or mediate) the relationships between DHEA-S levels and PAH risk or disease severity. Lower levels of DHEA-S may contribute to RV remodeling and fibrosis, with downregulation of NO and enhanced ET-1, explaining why lower levels DHEA-S are linked to overall PAH risk and greater severity of PAH and RV dysfunction leading to reduced 6MWD and worse functional class. These data establish a very strong scientific premise in both men and women to treat PAH patients with DHEA to improve clinical outcomes.

C4. Sex hormone manipulation in human PAH. In a small (n = 18) study, we have shown that the aromatase inhibitor anastrozole lowered E2 but had no effect on RV function (measured by echocardiographic parameters and BNP) and increased 6MWD.[15] Anastrozole reduced E2 but had no effect on circulating levels of DHEA-S (which may directly impact on NO, ET-1, the pulmonary vasculature and the RV); levels of DHEA-S and E2 are not related and DHEA-S had a direct effect on PAH risk and disease severity wholly independent of E2 levels. A larger, longer Phase II trial of anastrozole in PAH is planned (R01 HL134905; PI Kawut). While our pilot anastrozole study demonstrates the feasibility of sex hormone manipulation as a treatment strategy in PAH, there is no overlap in scientific premise or hypothesized mode of action for DHEA. For these reasons, studies of E2 blockade are complementary to our aims but do not address the therapeutic or mechanistic potential of DHEA in PAH.

Figure 7. CD146+/CD31+/CD45- cells from PAC tip sample

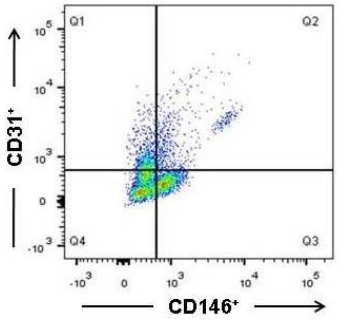
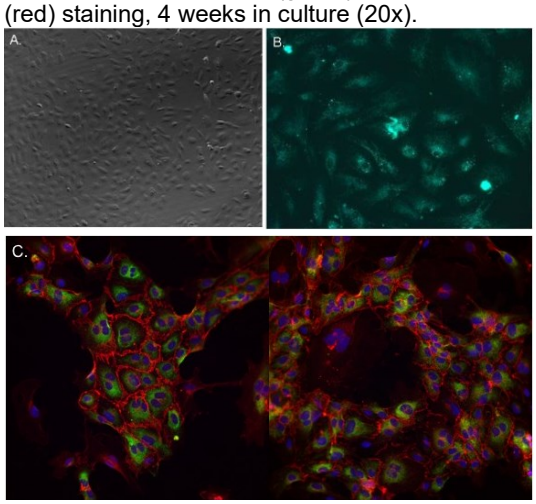


Figure 8. A. Phase microscopy image, 1 week in culture. B. AcLDL uptake of confluent cells, 2 weeks in culture. C. vWF (green), and VE-cad (red) staining, 4 weeks in culture (20x).



C5. Feasibility of collection and characterization of PAECs. Last, we sought to develop mechanistic experiments to explore sex-based differences in patients with PAH. We enrolled patients undergoing right heart catheterization for clinical purposes to isolate PAECs from pulmonary artery catheter (PAC) tips.[103] We first confirmed the presence of non-erythroid cells with counts varying from 25K–968K. We next confirmed PAEC phenotype ($CD146^+/CD31^+/CD45^-$ and acetylated low density lipoprotein [AcLDL] uptake, von Willebrand factor [vWF] and vascular endothelial-cadherin [VE-cad] staining) in additional samples (Figures 7-8). The NO Metabolomics core (University of Pittsburgh; collaborator Shiva) measured nitrite and nitrate levels in cells with confirmed PAEC phenotype ($CD146^+/CD31^+/CD45^-$ cells sorted; 8K cells) from culture media with excellent reproducibility (Table 4). Levels were consistent with those that mediate cellular signaling and suggest active secretion of NO.[104, 105] In our most recent experiments, we have isolated ~5 million cells in 3-4 passages and PAECs have remained viable up to 28 days (Figure 8C).

Table 4. NO metabolite levels

Sample	Nitrite, μM	Nitrate, μM
1	0.27	4.95
2	0.26	4.99
3	0.27	4.97
4	0.25	4.83
CV	<1%	<1%

CV=coefficient of variation; n=500 cells/sample

C6. Summary. The above studies are among the first to correlate sex and sex hormones (particularly DHEA) to pulmonary vascular and RV phenotype differences in men and women in both health and PAH. This proposal seeks to leverage a safe and available hormone treatment to gain further insight into 1) RV effects (a novel and critical end point in PH and PAH), 2) effects on two key PAH pathways *in vivo* and *in vitro* as a means for understanding sex-based differences in PAH, and 3) efficiency planning for a future Phase II parallel trial of DHEA as a novel treatment strategy in PAH.

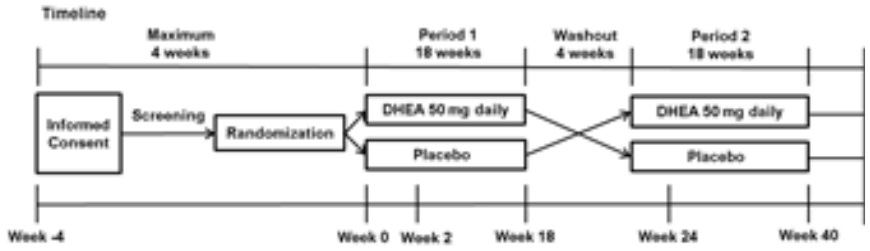
C7. Investigative team. The team assembled has expertise in clinical and translational research in PAH and RV failure. This group has collaborated for over five years in the areas of sex hormones, endothelial cell and vesicle biology, and cardiopulmonary function with funding and publications in these areas as well as participation in PAH clinical trials.[5, 101, 106-109] Collaboration with the NO Metabolomics core at Pittsburgh will greatly enhance measurement of NO end points in human and PAEC samples.

C8. Experimental Plan

C8.a. Study design. We propose a "proof-of-concept" crossover study to examine the safety and efficacy of 50 mg daily of DHEA for 18 weeks in patients with World Health Organization (WHO) Group

1 PAH (Figure 9). The primary outcome is RV longitudinal strain. Secondary outcomes include the effects of DHEA on markers of RV remodeling (**Aim 1**) as well as HRQOL, other PAH end points, side effects and safety. We will determine DHEA's effects *in vivo* in the cross-over trial (**Aim 2**) and *in vitro* on NO and ET-1 pathways in PAECs from subjects with PAH and whether these effects differ by sex **(Aim 3)**

Figure 9. Study protocol



C8.b. Scientific rigor. The selection of the cross-over design by definition eliminates between-subject variability when comparing drug to placebo and allows for a much smaller sample size than would ordinarily be necessary (e.g., in a parallel arm trial) to maintain the same power. Since each subject acts as their own control, it also prevents against the imbalance of other important factors related to rigor, namely age and race/ethnicity. However, differences in drug effectiveness by age, sex, etc. can still be assessed. This design will also allow us to examine patient-level responses in carefully selected end points (imaging, biomarker, functional, HRQOL) so that we may assess for potential interactions by age and race/ethnicity (as well as sex and PAH sub-type) and time by treatment effects while reducing variability and improving precision.

Study end points are subject to measurement error and missingness. Measurement error will be minimized by using validated methods with demonstrated precision in experienced laboratory and imaging cores with QC in place. Every effort will be made to minimize missing data via study procedures, close follow-up, and subject retention efforts. Bias will be minimized by masking patients, investigators, and personal and randomizing the treatment periods. While the proposed sample size is small, the cross-over design by definition optimizes statistical efficiency and minimizes confounding given each participant is their own control and treatment order is randomized. Multiple comparisons could result in Type I error, but our power calculations are based on distinct hypotheses about RV phenotype. Because of the cross-over design, small differences are likely to be detected making Type II error unlikely.

C8.c. Subjects. We will randomize 26 subjects stratified by sex (13 women, 13 men) and PAH type (idiopathic PAH vs. other) from the Rhode Island Hospital Pulmonary Hypertension Center (RIHPHC) to active treatment (Period 1), placebo (Period 2) or to placebo (Period 1), active treatment (Period 2) **(Aims 1, 2)**. PAH patients meeting the same inclusion criteria (Table 5) undergoing hemodynamics will be eligible for PAEC collection **(Aim 3)**.

C8.d. Inclusion/Exclusion criteria (Table 5). Patients on PAH therapy will be enrolled provided they have had no new PAH therapies introduced for 12 weeks (see Table 5 for specifics). We will include premenopausal women but will track menstrual cycle dates and measure sex hormones at each visit; physiologic variations should be balanced by the cross-over design (i.e., at least four to five menstrual cycles per 18 week treatment assignment). At least four weeks of clinical stability (no hospitalizations or titration of PAH therapies) will be required prior to randomization. Additional exclusion criteria speak to the elimination of other types of PH (#2, 5-6) or conditions in which DHEA is untested (#7-8) or has uncertain or potentially harmful effects (#1, 9-11). Indwelling (>3 months) levonorgestrel IUDs will be allowed. The progestin effect of hormone-releasing IUDs occurs primarily at the endometrium[147]. Serum hormone levels can be affected transiently (out to seven days postinsertion) but there is gradual decline over time which stabilizes. In a potential female subject with a long-term (3 months-5 years) hormone-containing IUD in place, systemic progestin levels are stable and, especially given the cross-over design, should not confound results.

Table 5. Inclusion and Exclusion Criteria

Inclusion Criteria
• mPAP ≥ 25 mmHg at rest, pulmonary capillary wedge pressure or left ventricular end-diastolic pressure ≤ 15 mmHg, and PVR > 3 Wood units at any time prior to study entry.
• Diagnosis of PAH that is 1) idiopathic, 2) heritable or 3) associated with connective tissue disease, congenital systemic-to-pulmonary shunt, porto-pulmonary hypertension, drug or toxin use
• Pulmonary function testing documenting forced expiratory volume in one second/forced vital capacity ratio ≥ 70% predicted, total lung capacity ≥ 70% predicted. o If TLC is mildly reduced (60%<TLC%<70%), computerized tomography (HRCT or non-HRCT) documenting no significant interstitial lung disease may be used to fulfill this requirement. • Chest tomography documenting no more than moderate parenchymal lung disease with clinician designated WHO I PAH and meeting both TLC and FEV1/FVC criteria. • Ventilation/perfusion (V/Q) testing documenting absence of thromboembolic disease (normal or low probability V/Q scan) o If no V/Q scan is available, a CT angiogram documenting the absence thromboembolic disease may be used, provided the subject meets diagnostic PAH criteria (as above).
Exclusion Criteria
1. Age < 18 years old
2. PAH associated with human immunodeficiency virus infection
3. Initiation of new background PAH therapy within 12 weeks of baseline visit
4. Significant dose change in background PAH therapy. The following ARE allowed: a. within 12 weeks of baseline visit:s i. planned titration of prostacyclin analogue, selexipag, or riociguat >20% ii. Plan to titrate to high dose sildenafil (e.g., 20mg to 80mg TID) iii. Transition to prostacyclin analogues/selexipag (e.g., IV to oral, inhaled to IV) iv. Increase in ambrisentan 5mg to 10mg or tadalafil 20mg to 40mg b. within 4 weeks of baseline visit: i. planned titration of prostacyclin analogue, selexipag, or riociguat <20% ii. change from commercial to generic (or generic to commercial) ERA or PDE5i iii. Interchange within drug class due to side effects (e.g., ERA to ERA, PE5i to PDE5i)
5. Untreated severe obstructive sleep apnea diagnosed by polysomnography
6. Evidence of left-sided valvular disease or systolic dysfunction on echocardiogram (≥ moderate mitral or aortic disease or LV ejection fraction ≤ 50%)
7. Glomerular filtration rate <40 mls/min/1.73m^2
8. Child-Pugh Class C cirrhosis
9. Untreated hypo- or hyper-thyroidism
10. Pregnant or breastfeeding
11. Active or planned use of hormonally active therapies with the exception of indwelling (>3months) levonorgestrel IUDs (see Appendix A for list of excluded medications and hormonal therapies).
12. History of breast, ovarian, uterine, testicular or prostate cancer
13. Current use of another investigational PAH therapy
14. Contraindication to MRI (e.g. metal device or fragment)
15. History of significant non-adherence or circumstance which would threaten ability to comply with cross-over design and study visit schedule

C8.e. Subject recruitment and retention. The RIHPHC services a catchment area of all of Southern New England and is the only center accredited by the Pulmonary Hypertension Association as a Comprehensive Care Center in the region. The RIHPHC cares for >400 patients and offers comprehensive patient care services including diagnostic imaging, hemodynamic evaluation, a dedicated PH step-down unit, an interdisciplinary care team, and four full-time providers. The PI and staff of RIHPHC meet weekly to screen patients for enrollment in active research protocols. Eligible patients will be approached by their PAH clinician and then by the PI or research staff. Following screening and informed consent, a maximum of four weeks may lapse before randomization (baseline visit) and eligibility in order to confirm clinical stability. New or recent changes to PAH meds will be reassessed at the baseline visit. Serum pregnancy tests will be obtained at

baseline and at week 24 (after crossover) in premenopausal subjects. Retention and complete follow-up will be assured with frequent call reminders 3-5 days prior to study visits, 24/7 accessibility of research support staff, study drug adherence logs and pill counts, and monetary compensation and parking passes for study visits.

PAH in men is rare, however based on our trial enrollment over the past three years (n = 52, 25% men) and our recent study of men[101] we anticipate recruiting 13 (50%) men during the study. Patient-level variation in end points as well as interactions by age, sex, race/ethnicity, and PAH sub-type will be assessed. We have met all of our enrollment targets for active studies, and consider this proposal a top priority for our center.

C8.f. Investigational therapy. We have already received an Investigational New Drug exemption (IND#129285) from the FDA for this study (see attached letter). We will purchase DHEA 50 mg daily tablets in a single batch from Green Mountain Pharmaceuticals (Lakewood, CO). Green Mountain is an Active Pharmaceutical Ingredient registered manufacturer with the FDA and has produced DHEA for ongoing trials at major centers including our institution (NCT01343771). Lots previously purchased from this manufacturer yielded >100% DHEA from an independent laboratory (FrontRangeLabs, Loveland, CO) (included with IND letter). We will also analyze and document purity in the batch purchased for this study. Study drug and placebo will be over-encapsulated by the Investigational Services Pharmacy (see letter of support). Dose, 18 week exposure and four week washout periods were chosen based on pharmacokinetics of DHEA, treatment duration required for RV changes, and early phase trial standards in endocrine and PAH.[36, 110-113] Changes in RV function occur after just 12 weeks of PAH therapy, justifying the planned exposure period.[36]

Table 6. Study protocol and end points

Timeline, week		**2**	**18**	**19-22**	**24**	**40**	**42**	**Unscheduled**
Study visit	Baseline	#1	#2	Washout	#3	#4		US
Phone call			X	X			X	
Medical history	X							
Medications	X	X	X		X	X		X
History and physical exam	X	X	X		X	X		X
Laboratory/safety								
CBC, Chem 7, hepatic	X	X	X		X	X		as indicated
Hormone levels	X	X	X		X	X		as indicated
Insulin levels	X	X	X		X	X		as indicated
Cholesterol levels	X	X	X		X	X		as indicated
Triglyceride levels	X	X	X		X	X		as indicated
End points								
Aim 1: Cardiac MRI	X		X			X		
Aim 1: NT-proBNP, galectin-3, RAGE, redox	X	X	X		X	X		
Secondary: SF-36v2, emPHasis-10, FC, 6MWD	X	X	X		X	X		
Aim 2: NO-related markers	X	X	X		X	X		
Aim 2: ET-1	X	X	X		X	X		
Study procedures								
Dispense study drug	X							
Medication adherence		X	X		X	X		
Adverse events		X	X	X	X	X	X	X

C8.g. Study protocol and burden. An 18 week supply of study drug will be dispensed following randomization at the baseline visit. Cardiac MRI for RV measures (Aim 1) will be assessed at baseline, week 18 and week 40, at the end of each period receiving active treatment or placebo. Laboratory (Aim1: NT-proBNP, galectin-3, RAGE, oxidative stress markers and those for safety; Aim 2: plasma NO-related markers and serum ET-1) and secondary end points will be assessed at baseline and all four study visits (Table 6). Data collection for history and physical exam, adherence, and adverse events will occur in the RIHPHC research room, followed by 6MWD in the RIHPHC hallway. Phlebotomy for study labs will be performed by trained personnel and cardiac MRI will be performed at the end of the visit in the MRI suite; labs and imaging will occur on the first floor of the same building where the RIHPHC is located. Subjects will complete the HRQOL measures on a tablet during downtime to r educe total visit time. We will offer a small meal after phlebotomy. Based on current protocols including similar tests we anticipate the total time for a study visit will be 90–120 min (visit #1, #3) and 210–240 min for visits with MRI (baseline, visit #2, #4).

C8.h. Drug and protocol adherence. Pill counts will be tracked at each visit. A phone call after visit #2 at week 18 will confirm plan for the washout period. A second call in the washout period prior to visit #3 will ensure start date for second course of study drug.

C8.i. Randomization and blinding. Randomization of drug sequence stratified by sex and PAH subtype (Period 1–Period 2; Period 2–Period 1) will be automated by a web-based program. Study personnel and

subjects will be blinded to treatment order allocation. DHEA and placebo will have identical packaging, taste, and smell. Study drug will be dispensed at the start of each treatment phase (weeks 0–18 [Period 1]; 22–40 [Period 2]) with phone calls as outlined.

C8.j. Safety monitoring. The research coordinator (Amy Palmisciano RN, BSN) and research assistant will be available 24/7 by phone as needed for side effects and adverse events. An unscheduled study visit can also be performed if lab abnormalities on safety labs require retesting. Provisions for an unscheduled study visit can be found in Table 6. RIHPHC clinicians and research staff including the study PI will be capable of breaking the blind in case of an adverse event. A data safety monitoring board (DSMB) consisting of three independent clinician scientists with expertise in cardiology, pulmonology and/or endocrinology and a biostatistician with clinical trial experience will be assembled to review enrollment targets, protocol deviations, violations, and adverse events as described below. Conference calls will occur throughout the study period and on an as needed basis for serious adverse events. Please see Section C8.m.6. for specific safety end points and **Data and Safety Monitoring** document for further details.

C8.k. Drop-out. Subjects may end participation by completion of the trial, loss-to-follow-up, withdrawal of consent, or death. In the last three RCTs conducted at our center (n = 21) we have had no patients lost to follow-up (rare in this patient population) and two patients (11%) withdraw. In our power calculations, we have anticipated a 10% drop-out rate; any subjects who need to withdraw from the study will continue study assessments whenever possible and be analyzed in an intent-to-treat fashion.

C8.l. Outcomes for the crossover trial. The primary outcome will be changes in longitudinal strain with DHEA **(Aim 1)**. Secondary outcomes will include RVEF, NT-proBNP, galectin-3, RAGE, MPO and sNOX2-dp levels **(Aim 1)**, sex hormone levels, HRQOL, WHO functional class, 6MWD, side effects, and safety of DHEA in PAH. We considered using right heart catheterization as a study end point but given the cross-over design (which has benefits in terms of power and precision) this would require three right heart catheterizations which poses undue burden on (and risk to) participants and a threat to feasibility. Additional end points (e.g., HRQOL) were selected based on prior DHEA trials demonstrating efficacy in similar disease states with high relevance to PAH. **Aim 2** outcomes will include changes in NO and ET-1 molecules with DHEA treatment.

C8.l1. Aim 1: Longitudinal strain and RVEF. We have a core MRI facility with a Siemens 1.5T Aera with full Advanced Cardiac Package and XJ Gradients (33 mT/m @ 125 T/m/s) that provides research grade studies at a greatly reduced cost. A single reader (Michael Atalay, MD, PhD) will be blinded to subject and study visits and will read studies in randomly sequenced batches; 10% of the studies will be re-read by the same reader and 10% of scans will be re-read by a second reader for intra-reader and inter-reader reliability estimates. Dr. Atalay's research focuses on cardiac MRI and he is experienced in RV imaging in PAH. A standardized MRI protocol is proposed:

1) Quantification of end-diastolic volume (EDV), end-systolic volume (ESV), EF, and mass. Short-axis steady state free precession from the base of the heart through the apex (Figure 10). All analyses will be performed using commercially available cardiac MRI software (CVI42, Circle Cardiovascular Imaging). The endocardial and epicardial borders of both ventricles will be traced manually on short axis cine images at end-diastole and end-systole. EDV and ESV will be calculated using Simpson's rule by summation of areas on each slice multiplied by the sum of slice thickness and image gap. Ventricular EFs will be calculated by dividing respective stroke volumes (EDV-ESV) by EDVs. RV and LV mass will be determined at the end-diastole phase as the difference between end-diastolic epicardial and endocardial volumes X heart specific gravity (1.05 g/cm^3).[114]

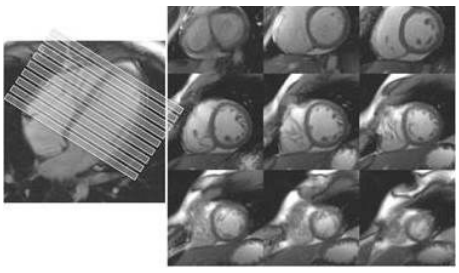


Figure 10. Short axis cine images for volume measurements including RVEF

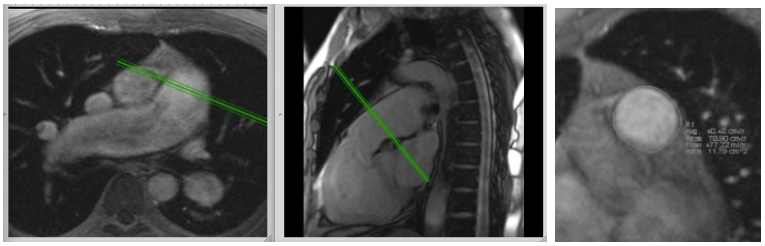


Figure 11. Through-plane phase contrast for main PA distensibility

2) Quantification of pulmonary insufficiency, independent quantification of RV stroke volume and measurement of main PA cross-section through the cardiac cycle (distensibility). Breath-hold through-plane phase contrast imaging with a velocity encoding gradient (VENC) of <120 cm/s (larger if aliasing

present) in the main PA ~2-3 cm above the pulmonary valve plane, with imaging plane oriented orthogonal to the main PA (Figure 11). Free-breathing phase contrast imaging in the same plane and VENC with averaging over 4 respiratory cycles.

3) Longitudinal and circumferential strain will be determined using standard cine imaging and MTT software (version 6.0, Toshiba, Japan). This program utilizes a pixel-matching algorithm to define angle-independent motion vectors from multiple tracking points for location of identical voxels in serial frames (Figure 3). Algorithm will be repeated twice for each subject and include biventricular borders throughout the cardiac cycle. Peak systolic segmental strain will be extracted from inner, mid, and outer walls.

C8.l2. Aim 1: Markers of RV Phenotype. Blood via venipuncture will be collected by trained staff. Samples will be processed and stored at -80ºC. Serum NT-proBNP levels will be measured using a FDA approved commercially available immunoassay (Roche Diagnostic Elecsys proBNP Assay, Indianapolis, IN) with excellent precision (intra- and inter-assay CV of <6.1%) and a track record in PAH.[115, 116] Serum galectin-3 levels will be measured by an enzyme linked immunoassay (ELISA) with an intra-assay CV of 3.4% (BG Medicine, Waltham, MA). Soluble RAGE levels in plasma will be measured by an ELISA (R&D Systems, Minneapolis, MN) with excellent precision (intra-assay CVs 4.8% – 6.2%; inter-assay CVs 6.7% – 8.2%). Serum MPO levels will be measured by a commercially available ELISA (CardioMPO Test, Prognostix, Cleveland, Ohio) and sNOX2-dp by ELISA using previously published methods, both with excellent precision.[72]

C8.m. Secondary outcomes for the crossover trial

C8.m1. HRQOL. Patient-reported outcomes as assessed by HRQOL measures provide important information not captured by traditional surrogates and especially relevant in PAH, a progressive disease without cure treated with burdensome and side effect-laden therapies. The **SF-36v2** is a widely used instrument that provides information about HRQOL along 8 domains condensed into physical and mental component summary scores. The SF-36 and its updated version SF-36v2 has been validated in chronic diseases including PAH, has been used as a secondary end point in RCTs of approved PAH therapies, and tracks with survival.[117-123] **emPHasis-10** is a PAH-specific HRQOL tool that is uni-dimensional and pragmatic (10 items). It is sensitive to clinical changes in PAH and has been translated into multiple languages with cultural and linguistic validation.[124, 125] Subjects will be asked to complete the SF-36v2 and the emPHasis-10 at each research visit (Table 6). Changes from baseline in domain-specific scores and summary scores for the physical and mental components for the SF-36v2 and the summary score for emPHasis-10 will be assessed.

C8.m2. WHO Functional class. Functional classification drives initial choice of therapy and is a major treatment goal in PAH.[126] WHO class is derived from the New York Heart Association classification: Class I is no limitations; Class II is slight limitations in physical activity and no symptoms at rest; Class III is symptoms with minimal activity; Class IV is symptoms at rest. Clinicians will assess functional class at each study visit.

C8.m3. 6MWD. Change in 6MWD has been the primary end point for almost all Phase III trials and the basis for drug approval in PAH.[127] Testing will be performed by blinded and trained personnel according to standardized procedures.[128] We will minimize variability by administering the test in the same corridor for all subjects, avoiding a “warm-up” period, and using standard phrases of encouragement; intra-class correlation coefficients were 0.95 – 0.98 from a recently completed RCT in our center (NCT01545336).

C8.m4. Sex hormone levels. We will measure serum sex hormone levels at baseline and with each study visit to capture overall changes in the hormonal mileau during active treatment and placebo phases using highly sensitive assays with excellent QC as we have previously reported.[15, 101] DHEA-S (more stable than DHEA in biosamples) will be measured on the Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Deerfield IL).

C8.m5. Additional signaling molecules of interest. Given experimental PH models have linked DHEA to important signaling pathways in the immune and fibroproliferative components of PAH (e.g., IL-6, MAPK, STAT3), we will investigate whether DHEA treatment impacts serum IL-6 and soluble IL-6 receptor levels using commercial ELISAs.[19, 20, 65, 129] PAEC experiments will also assess MAPK and STAT3 signaling (Section C9.).

C8.m6. Side effects and safety. Study personnel will assess side effects and adverse events by body system during each visit and in phone calls during the washout phase and end of study call at week 42. We will specifically query participants about potential known side effects of DHEA, such as androgenic symptoms (change in skin/greasy skin, acne, alopecia and/or hirsutism) and we also assess HRQOL from these potential side effects as outlined above with the SF-36v2. Laboratories to assess for safety will include: complete blood count, complete metabolic panel, glucose levels, insulin levels, liver function tests, high density lipoprotein, low

density lipoprotein, total cholesterol and triglyceride levels using standardized assays. While we do not anticipate drug-related adverse events, in all cases the referring and/or primary care physician will be notified in writing of the adverse events, the subject's status, and receive a copy of laboratory abnormalities, as appropriate. Study drug will be continued for mild side effects such as androgenic symptoms. A detailed monitoring plan will be submitted to the local IRB before the trial begins in accordance with NIH/NHLBS policy for early phase trials and will include mechanisms for adverse event reporting to the IRB, the FDA, and the NIH (see Human Subjects Protection). No interim analysis is planned for this pilot study.

C8.m7. Aim 2: NO-related molecules. NO synthesis will be assessed by the NO Metabolomics core facility (University of Pittsburgh; collaborator Shiva). Plasma nitrite and nitrate levels will be quantified using tri-iodide-based reductive chemiluminescence.[105, 130, 131] Whole blood will be immediately processed with a readily available precooled centrifuge to 4ºC after the addition of nitrite preservation.[130] Care will be taken to avoid sample exposure to light and to use nitrite-free equipment. The specific contribution of nitrite will be calculated by subtracting nitrosocompounds from the total chemiluminescence signal after treatment with acidic sulfanilamide. We additionally propose to measure interrelated molecules in the NO pathway known to be abnormal in PAH including cGMP levels and S-nitrosohemoglobin (SNO-Hb), a major regulator of NO's interaction with vascular endothelium.[132-135] cGMP will be measured via immunoassay (Biomedical Technologies, Inc, Madrid, Spain).[136] SNO-Hb levels will be measured using chemiluminescence as for nitrites and nitrates; these methods have been well-validated by the NO Metabolomics core facility.[131]

A potential pitfall is that the direct measurement of steady state NO is difficult due to endothelial and cellular metabolic flux and rapid half-life. As such the sum of nitrates and nitrites are not necessarily reflective of total NO synthesis activity and may capture changes in diet or external contamination, for example.[137] The measurement of nitrates is subject to such interference, whereas as much as 90% of circulating nitrites in plasma is the result of the L-arginine-NO production pathway. The NO Metabolomics core facility at the University of Pittsburg has extensive experience in assessing pulmonary vascular NO and employs state-of-the art approaches to ensure accuracy and precision known to be more sensitive for NOS signaling than other methods (especially from banked samples) including electron paramagnetic resonance.[130, 138]

C8.m8. Aim 2: ET-1. Serum ET-1 will be measured via immunoassay (QuanitGlo, R&D Systems, Inc, Minneaopolis, Minn) with intra-assay CVs of 2.6 – 3.4% and inter-assay CVs of 4.6 – 8.9%.

C8.n. Data collection and management. Our clinical research coordinator will oversee data collection and management. She has extensive experience working in pulmonary/critical care clinical-translational research and specifically in PAH for the past four years. All data will be collected in a secured fashion with paper case report forms and immediately transposed to an electronic system (REDCap) to ensure back-up. Data will be stored on a secure server. Plan for data sharing with the University of Pittsburgh is described in the Plan for Resource and Data Sharing and Human Subjects Protection documents. The PI will be responsible for data security and cleaning; analysis will be completed by G. Baird, PhD who has collaborated closely with the PI.

C8.o. Analysis plan for the crossover trial. Demographics and clinical data will be provided, stratified by sex as well as PAH subtype. This data will also be tracked for screen failures and drop-outs. Continuous data will be expressed as medians and ranges and categorical data will be expressed as frequency and percentages. Analyses will follow the intention-to-treat paradigm. All end points will be modeled using generalized estimating equations (GEEs), nesting repeated measures within patient and including terms for sex, age, race/ethnicity, PAH-subtype, randomization, and time point, along with all 2-way and 3-way interactions in which carry-over or period effects will be considered. Distribution will be chosen based on inspection of model residuals. Classical sandwich estimation will be used to adjust for model misspecification after maximizing the appropriateness of distribution selected. Orthogonal linear estimates will be constructed within GEEs to test each hypothesis (see Aims) and presented both as the percentage change and absolute change in RV measures **(Aim 1)**, changes in NO products and ET-1 **(Aim 2)**, and secondary end points. No adjustment for multiple comparisons will be made given the pilot nature of the study. The main effect of DHEA and interactions of DHEA with sex (as well as age, race/ethnicity, PAH-subtype) will be tested within models, with use of visualizations including individual scores for exploratory analysis. Hypotheses will be two-tailed.

C8.p. Sample size and power for the crossover trial. We anticipate a 10% drop-out rate and calculated power based on an anticipated sample size of 24 subjects (12 men, 12 women). As this is a pilot study, detection of a signal (even if not statistically significant) and "proof of concept" that DHEA treatment may improve RV phenotype and alter major PAH regulators would encourage the pursuit of a

larger Phase II parallel arm trial. Time by treatment and PAH subtype by treatment interactions will also be considered as part of 3-way interactions terms in GEE.

C8.p1. Aim 1: Power to detect differences in RV longitudinal strain. We will have excellent power to detect clinically relevant changes in RV strain (α = 0.05)(Table 7). A ≥ 5% improvement in RV strain (measured by echocardiography; less precise than MRI) occurs with approved PAH therapies and predicts clinical stabilization and survival in PAH.[56] We have more than enough power to detect this estimate, and effects even as small as 3%. We need this number of patients in order to perform sex-specific analyses (and test for treatment x sex interaction), where we will have 80% power to detect a 4% difference in sex-specific analyses with the presence of an interaction. Power for treatment x PAH subtype interactions will mirror these estimates. We will also have sufficient power to detect changes which approach clinical relevance in RVEF[61, 139] and NT-proBNP[140] (α = 0.05) (Table 8).

Table 7. Power calculations for RV strain (N = 24)

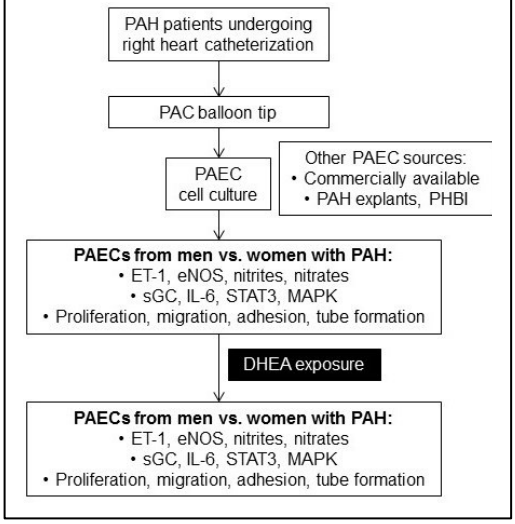
	RV strain difference, %	Power
No interaction	3.0	89%
	5.0	99%
	7.0	100%
Interaction	4.0	80%
	5.0	94%
	7.0	100%

Table 8. Power calculations for RVEF and NT-proBNP (N = 24)

	RVEF, %	Power	NT-proBNP, mg/L	Power
No interaction	5.0	80%	200	98%
Interaction	8.0	80%	200	80%

C8.p2. Aim 2: Power to detect differences in nitrate and ET-1 levels. We will have excellent power to detect small (0.05 μM) differences in nitrite levels with DHEA treatment based on conservative previously observed estimates (α = 0.05).[25, 141] We will have excellent power to detect small (well-less than 2 pg/mL) differences in ET-1 levels with DHEA treatment (α = 0.05). Changes of this magnitude would be consistent with clinically relevant changes in ET-1 with ERAs.[142]

Figure 12. Schematic for Aim 3



C9. Aim 3: PAEC response to DHEA. In order to minimize burden of the trial participants (and to guarantee more catheter tips), PAECs from men and women undergoing right heart catheterization at the RIHPHC as part of PAH diagnosis or treatment and meeting the same inclusion/exclusion criteria for the cross over trial will be included. As with our preliminary data, these experiments will be performed in our Centers of Biomedical Research Excellence in Stem Cell Biology (PI: Quesenberry, P20 GM103468) and Cardiopulmonary Vascular Biology (PI: Rounds; Laboratory Core PI: Harrington, P20 GM103652). We will also examine the effect of DHEA on PAECs obtained from two additional sources: 1) commercially available human PAECs, 2) those obtained from the distal pulmonary circulation of PAH lung explants available through the Pulmonary Hypertension Breakthrough Initiative (PHBI) (PI Geraci, R24 HL123767; see letter of support).

We will isolate PAECs and assess NO and ET-1 production at baseline and following exposure to DHEA (Figure 12). Following catheterization, the PAC tip will be placed into 1 well of a 24 well plate, with Attachment Factor Solution (Cell Applications, Inc, San Diego, CA) and media (EndoGRO, Millipore Sigma, Billerica, MA). The PAC tip will be washed with fresh media every 2 days and previous media from the tip well will be moved into a new well in the 24 well plate. Cells will be grown in primary culture to confluence (~150K-200K cells) over 2-3 weeks. Cells will then be treated with Triple Express (ThermoFisher Scientific, Waltham, MA) and transferred to T-25 and then T-75 flasks over 4-5 days; we anticipate ~5 million cells in 3-4 passages. In the event that we are unable to culture a robust number of PAECs using the above protocol, conditioned media charcoal-stripped[143] of all steroid hormones will be used.

1) NO and ET-1 synthesis. Cell lysate and culture media from cultured PAECs passage 3-5 will be snap frozen in liquid nitrogen and stored at -80ºC. ET-1 and cGMP will be measured by ELISA and nitrite and nitrate levels will be quantified using tri-iodide-based reductive chemiluminescence (See Section C8.m7). Expression of eNOS, soluble guanylyl cyclase (sGC) α and β, IL-6, STAT3, and MAPK will be measured with standard RT-PCR and Western blot methods using commercial nucleic acid probes and antibodies.[144]

2) Cell treatment. Cells will be incubated at 37°C in media in the presence or absence (controls cells) of DHEA at final concentrations of 1, 10, and 100 μM (Sigma-Aldrich, St. Louis, MO). Initial experiments will be conducted using commercially available PAECs to determine optimum cell concentration and exposure times that will provide consistent assessment of NO and ET-1 synthesis. Cell proliferation, migration, adhesion, and tube formation will be characterized before and after exposure to DHEA using standardized protocols

performed in our laboratory[145] with concurrent confirmation of PAEC phenotype. We will also determine the effect of DHEA in commercially available disease free PAECs. These conditions will then be used in experiments to assess DHEA's effects on NO and ET-1 synthesis in PAECs from both sexes with PAH. All experiments will be repeated with 1) exposure to E2 for comparison and 2) in PAECs from lung explants to determine if distal and proximal PAECs respond in a similar way to DHEA.

C9.a. Analysis Plan Aim 3: PAEC response to DHEA. Wilcoxon signed-rank tests and analysis of variance will be used to compare ET-1 and eNOS expression and nitrite and nitrate levels before and after DHEA treatment and across cells from men and women.

C10. Study timeline. Based on prior recruitment rates and fractions, we will be able to enroll 4-7 subjects per year as outlined in the study timeline. A five year mechanism is proposed and is needed in order to ensure adequate inclusion of men and completion of the protocol for subjects recruited later in the study period to allow adequate time for follow-up, biomarker and data analysis (which cannot be completed until all subjects enrolled later in the study complete the 42 week crossover trial protocol).

Table 9. Study timeline

	Yr 1	Yr 2	Yr 3	Yr 4	Yr 5
Start-up: Drug purity testing	Mos 1-6				
Relational trial database buildout	Mos 1-6				
Policies and Procedures, IRB approval	Mos 1-6				
Aim 1,2: Cross-over enrollment	n = 4	n = 7	n = 7	n = 6	n = 2
Aim 3: Commercial cells, PHBI	X	X	X	X	
Aim 3: PAECs, patient catheterizations	n = 15	n = 15	n = 15	n = 15	n = 15
Lab/results analysis				X	X
Manuscript/grant preparation				X	X

C11. Expected results

C11.a. Aims 1 and 2. Active treatment with DHEA will be associated with an improvement in RV strain pattern, lower ET-1 levels and higher nitrite and nitrate levels. Markers of maladaptive RV hypertrophy including oxidative stress will also be reduced. Sex will modify these relationships, with women demonstrating a more robust response to DHEA.

C11.b. Aim 3. NO and cGMP synthesis will be higher and ET-1 secretion will be lower in PAECs from women with PAH than PAECs from men, respectively. Treatment with DHEA will increase eNOS expression and nitrite/nitrate levels and decrease ET-1 secretion. Female cells will demonstrate a more robust and distinct response to DHEA than male cells and E2 exposure respectively.

C12. Potential problems and alternative approaches

C12.a. Aims 1 and 2: Clinical trial completion and end point evaluation. Enrollment: A major threat to clinical studies in a rare disease is low enrollment. The RIHPHC has an extensive track record of participation in NIH funded efforts of hormonal modulation in PAH where we have met and surpassed recruitment goals (P20 GM103652 [PI:Ventetuolo] and K24 HL103844 [PI: Kawut; NCT01545336]).[146] We have tried to minimize burden, provided incentives, and prioritized this trial over invitations to participate in others to ensure adequate recruitment. Drop out will be minimized by study procedures and follow-up ensured as PAH patients require highly specialized care available regionally only at the RIHPHC. Study endpoints: In the event that DHEA has no effect on our primary end point, a number of other secondary and exploratory end points have been incorporated which capture alternative markers of interest (e.g., oxidative stress) and will serve to shed light on sexual dimorphism in PAH. We considered measurement of central hemodynamics a lower priority given our interest in studying the direct effects of DHEA on the RV, and felt the burden from repeat right heart catheterizations would threaten feasibility. Intervention: We selected a lower dose of DHEA (50 mg) than given in the prior trial of COPD-PH (200 mg) because the majority of RCTs have used the 50 mg dose with no serious adverse events and minimal side effects.[73, 79] We considered a dose-finding study, but the 50 mg dose has been very well-established as sufficient to increase DHEA-S levels without intolerable side effects. The 50 mg dose has been associated with improvements in biomarkers in atherosclerosis and HRQOL in lupus (as proposed here). A highly pure compound will be verified. It is possible that DHEA may work via alternative mechanisms to those proposed; alternative biomarkers may be tested from banked samples from study visits to be further developed in our cell-based experiments.

C12.b. Aim 3: PAEC response to DHEA. This aim will be completed in PAH patients undergoing routine right heart catheterization, which may or may not include patients included in the crossover trial and therefore may

constitute a “missed opportunity” to study results of *in vivo* controlled DHEA exposure *in vitro*. We did not incorporate catheterization into the study protocol in an effort to minimize burden but did restrict eligibility for this aim to match inclusion/exclusion criteria for the trial. We perform dedicated right heart catheterizations in 40 – 60 patients yearly, providing ample opportunities to obtain PAECs from PAH patients. Over the last twelve months since this protocol was initiated, PAEC samples have been collected in 57 patients. The PAECs collected (and their response to DHEA exposure) may not represent the phenotype of PAECs from small resistance vessels in PAH (which will also be included for comparison, Section C9). PAH patients have changes in proximal PAs which contribute to circuit compliance and ventricular coupling[147], however, making the proposed experiments relevant. Moreover, recent studies using induced pluripotent stem cells from dermal fibroblasts of PAH patients showed that derived endothelial cells function the same as distal PAECs isolated from explanted lungs.[148] PAECs obtained from lung explants represent end-stage PAH, whereas our method captures PAECs from patients at variable stages of disease. Finally, this is a very novel approach with which we have become increasingly facile in a disease state in which tissue availability is extremely limited.

C13. Summary. This pilot double-blind cross-over trial will test the efficacy and safety of DHEA in men and women with PAH and will characterize the interaction of sex- and sex-hormones with major pathologic drivers of pulmonary vascular and RV dysfunction. Complementary *in vitro* experiments will offer additional insights into mechanisms of sexual dimorphism in PAH. The first study of its kind, we will build a foundation for sex- and hormone-based treatment precision in this rare and progressive disease.

Amendments and Addendums:

Amendment 1 – PAEC Biobanking and RNA Sequencing

Pulmonary arterial cells (PAECs) collected from catheter tips may undergo RNA sequencing as part of an advanced characterization protocol, representing a continuation of the aims of Aim 3 above. The PAECs for RNA Sequencing will be sourced from the current study’s subjects and from a previous biobank (PI: Klinger, IRB Committee #: 016311). Furthermore, all PAEC cultures will be preserved for future, unknown testing and stored in accordance with the Lifespan HRPP Specimen banking guidelines.

Amendment 2 – Biobanking for Aim 1 and 2 (clinical trial samples)

A portion of blood collected (in the form of plasma and serum) under Aim 1 and 2 of the protocol (“the DHEA clinical trial”) will also be banked. No additional blood will be collected. We will bank the remnant samples after protocol laboratory testing has been performed.

Amendment 3 – Unscheduled Visit Provision, Inclusion/Exclusion Clarification and SF-36v2

1. Added provisions for an unscheduled study visit in case of clinically significant safety lab abnormalities. Details can be found in Table 6 and section C8.j.
2. This amendment also clarifies three inclusion criteria:
   A. If TLC is mildly reduced (60%<TLC%<70%), computerized tomography can be used in order to rule out any clinically significant interstitial lung disease and allow for inclusion.
   B. Changes “absence” of parenchymal lung disease to “no more than moderate” disease with the provision that the treating physician has deemed the patient’s underlying PAH as unrelated or out of proportion to lung disease (i.e. patient has WHO group 1 PAH) and the patient meets PFT criteria. The PI will verify the validity of all diagnoses.
   C. If no V/Q scan is available and meets study diagnostic criteria for WHO 1 PAH, a negative CTA Pulmonary Embolism can be used instead.

   The above three clarifications put the study in congruence with the diverse phenotypic realities of the PAH patient population. We do not believe these changes will have any impact on the scientific rigor of this study.

   All changes are detailed in Table 5.
3. One exclusion criteria has been added excluding potential subjects with “a history of significant non-adherence or circumstance which would threaten ability to comply with cross-over design and study visit

schedule".

4. Clarified the version of the SF-36 used in this study to be the SF-36v2. All discussion detailing the application of the SF-36 to the study holds true for the SF-36v2.

Amendment 4 – Addition of blood sample collection for AIM 3 cohort during RHC

In addition to collecting right heart catheter tips, we will collect up to 30 mL (2 tablespoons) of blood at the time of right heart catheterization from all consented subjects enrolled in Aim 3. Platelets and peripheral blood mononuclear cells (PBMCs) will be isolated and analyzed for pathways of metabolomics and for Xist RNA for X chromosome inactivation.

Protocol Addendum 1

Provisions for Study Drug Extension due to COVID-19

For subjects currently enrolled in EDIPHY, we may extend the active treatment period an additional four weeks (Total time of treatment = up to 22 weeks) to complete in person study visits and obtain end points which cannot be obtained virtually/by televisits (i.e., six minute walk distance, cardiac magnetic resonance imaging). This strategy optimizes subject safety (by moving the window out another four weeks, to avoid an in person study visit if conditions so dictate) while minimizing data loss, the chance of "wasted" participation, and our ability to detect efficacy which is best done with a subject on study drug. Based on a prior clinical trial of 381 subjects with systemic lupus erythematosis, which was conducted with a higher dose of DHEA for one year's duration with no serious adverse events (Petri et al., Arthr Rheum 2004), we believe this extension of only four weeks poses no additional risk to subjects.

The changes follow:

1. Continue study drug until 6MWT and cMRI can be completed (up to an additional 4 weeks); remainder of end points will be assessed virtually on their due date and then coincident with 6MWT and cMRI
2. Scheduling
    a. Active subjects will be contacted proactively to inform them of any relevant updates to study conduct and address any concerns.
    b. The consent addendum will be sent to their residence and reviewed by phone with a study staff member. If willing, study staff will ask the subject to send the completed form back. Study staff will then sign and send a copy of the consent to the subject.

Visit Procedures

1. Safety Labs and Laboratory Endpoints
    a. If an off-campus laboratory is available to a subject, safety labs will be drawn and reviewed via remote medical record review.
    b. Alternatively, if the subject has a previously scheduled clinical visit in the PH Center, safety labs and study labs will be ordered and drawn at this clinic visit.
2. 6MWT
    a. If a subject has a previously scheduled clinical visit in the PH Center, a 6MWT will be performed at this visit.
    b. Otherwise, study drug will be continued for up to 4 additional weeks until 6MWT can be obtained
3. cMRI
    a. Study drug will be continued for up to 4 additional weeks until cMRI can be obtained

Other Study Drug Details

1. Study Drug Dispensing
    a. Provisions for remote study drug dispensing are being discussed with Lifespan research pharmacy should any subject transition for Period 1 to Period 2.

b. Research pharmacy will be notified about need for possible additional duration of study drug
2. Follow-up in extension period
    a. Study staff will continue to follow-up and collect adverse events or any other problems the subject may have.
    b. When a cardiac MRI and 6MWT is able to be scheduled, safety labs will be collected as a "Unscheduled Visit" at this time.
    c. The Week 19 phone call will occur 1 week after you complete the extension period (if in Treatment Period 1).
    d. The Week 21 phone call will occur 3 weeks after you complete the extension period (if in Treatment Period 1).
    e. The Week 42 phone call will occur 2 weeks after you complete the extension period (if in Treatment Period 2).

Protocol Addendum 2

Provisions for COVID-19 Remote Follow-up

In preparation of further COVID-19 precautions, this addendum details provisions to the EDIPHY protocol if remote subject visits become necessary. It seeks to minimize data loss while also ensuring subject safety. Until this point, subject participation will continue per protocol with the addition of ORA/RPO recommended screening procedures.

The changes follow:

<u>If remote visits become necessary:</u>

1. Continue study drug until 6MWT and cMRI can be completed (up to an additional 4 weeks); remainder of end points will be assessed virtually on their due date and then coincident with 6MWT and cMRI
2. Scheduling
    a. Active subjects will be contacted proactively to inform them of any relevant updates to study conduct and address any concerns.
    b. If necessary, a remote phone or video visit will be scheduled with the subject.
    c. The consent addendum will be sent to their residence and reviewed by phone with a study staff member. If willing, study staff will ask the subject to send the completed form back. Study staff will then sign and send a copy of the consent to the subject.

Visit Procedures

1. Safety Labs and Laboratory Endpoints
    a. If an off-campus laboratory is available to a subject, safety labs will be drawn and reviewed via remote medical record review.
    b. Alternatively, if the subject has a previously scheduled clinical visit in the PH Center, safety labs and study labs will be ordered and drawn at this clinic visit.
2. 6MWT
    a. If a subject has a previously scheduled clinical visit in the PH Center, a 6MWT will be performed at this visit.
    b. Otherwise, study drug will be continued for up to 4 additional weeks until 6MWT can be obtained
3. cMRI
    a. Study drug will be continued for up to 4 additional weeks until cMRI can be obtained
4. Quality of Life Surveys
    a. SF-36v2, emphasis-10 and satisfaction surveys will be administered over the phone and entered directly in REDCap. Alternatively, if the subject feels comfortable, a remote REDCap survey link will be sent to their personal email and completed directly.
5. Physician contact

    a. In the absence of an in-person exam, a study physician will remotely perform a review of systems with the subject and assign a WHO functional class at the time of the remote visit.
6. Adverse Event, Concomitant Medication and Other Subject Review
    a. All new or active events, changes in medication and other relevant information will be recorded at the time of the remote visit per standard procedure.
    b. Study drug adherence will also be reviewed with the subject and recorded.
7. Subject Compensation
    a. A check request form will be completed by study staff and the subject will receive a check commensurate with assigned visit amount within 4-6 weeks.
8. Medical Monitor Review
    a. The independent medical monitor will review all changes necessitated by a shift to remote visits and review the safety information collected per standard procedure.
9. Documentation
    a. Hardcopies of essential study documentation (without PHI) will be brought home with an assigned staff member in preparation for remote visit.
    b. Documents will be prepared for all active subjects proactively in preparation for any emergent changes.

Other

1. Study Drug Dispensing
    a. Provisions for remote study drug dispensing are being discussed with Lifespan research pharmacy should any subject transition for Period 1 to Period 2.
    b. Research pharmacy will be notified about need for possible additional duration of study drug
2. Follow-up in extension period
    a. Study staff will continue to follow-up and collect adverse events or any other problems the subject may have.
    b. When a cardiac MRI and 6MWT is able to be scheduled, safety labs will be collected as a “Unscheduled Visit” at this time.
    c. The Week 42 phone call will now occur 2 weeks after completion of the extension period

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