



ORIGINAL RESEARCH

Mechanistic Phase II Clinical Trial of Metformin in Pulmonary Arterial Hypertension

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BACKGROUND: Metabolic dysfunction is highly prevalent in pulmonary arterial hypertension (PAH) and likely contributes to both pulmonary vascular disease and right ventricular (RV) failure in part because of increased oxidant stress. Currently, there is no cure for PAH and human studies of metabolic interventions, generally well tolerated in other diseases, are limited in PAH. Metformin is a commonly used oral antidiabetic that decreases gluconeogenesis, increases fatty acid oxidation, and reduces oxidant stress and thus may be relevant to PAH.

METHODS AND RESULTS: We performed a single-center, open-label 8-week phase II trial of up to 2 g/day of metformin in patients with idiopathic or heritable PAH with the co-primary end points of safety, including development of lactic acidosis and study withdrawal, and plasma oxidant stress markers. Exploratory end points included RV function via echocardiography, plasma metabolomic analysis performed before and after metformin therapy, and RV triglyceride content by magnetic resonance spectroscopy in a subset of 9 patients. We enrolled 20 patients; 19/20 reached the target dose and all completed the study protocol. There was no clinically significant lactic acidosis or change in oxidant stress markers. Metformin did not change 6-minute walk distance but did significantly improve RV fractional area change ($23\pm 8\%$ to $26\pm 6\%$, $P=0.02$), though other echocardiographic parameters were unchanged. RV triglyceride content decreased in 8/9 patients ($3.2\pm 1.8\%$ to $1.6\pm 1.4\%$, $P=0.015$). In an exploratory metabolomic analysis, plasma metabolomic correlates of $\geq 50\%$ reduction in RV lipid included dihydroxybutyrate, acetylputrescine, hydroxystearate, and glucuronate ($P<0.05$ for all). In the entire cohort, lipid metabolites were among the most changed by metformin.

CONCLUSIONS: Metformin therapy was safe and well tolerated in patients with PAH in this single-arm, open-label phase II study. Exploratory analyses suggest that metformin may be associated with improved RV fractional area change and, in a subset of patients, reduced RV triglyceride content that correlated with altered lipid and glucose metabolism markers.

REGISTRATION: URL: <http://www.clinicaltrials.gov>; Unique identifier: NCT01884051.

Key Words: insulin resistance ■ metformin ■ pulmonary arterial hypertension ■ right ventricle

Metabolic dysfunction resembling features of insulin resistance is prevalent in pulmonary arterial hypertension (PAH) and likely contributes to both pulmonary vascular disease and right ventricular (RV) failure, the primary cause of death in PAH. Animal models of PAH demonstrate increased pulmonary

vascular and right ventricular (RV) glycolysis,¹⁻⁶ impaired fatty acid oxidation,^{7,8} and insulin resistance that is associated with more advanced pulmonary vascular disease,^{9,10} accumulation of myocardial lipid, and impaired RV function.¹¹ Observational studies in humans with PAH corroborate the clinical relevance of these

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CLINICAL PERSPECTIVE

What Is New?

- Although metabolism and insulin resistance have been recognized in pulmonary arterial hypertension, there have been no trials of therapies targeting these alterations in this deadly disease.
- In this pilot trial, we found that metformin is safe in patients with pulmonary arterial hypertension and that, in secondary analyses, metformin may improve right ventricular function and non-invasive markers of right ventricular lipotoxicity that tracked with changes in the plasma metabolome.

What Are the Clinical Implications?

- This trial provides a rationale for further study of metformin in pulmonary arterial hypertension and suggest that the plasma metabolome may be useful in identifying patients with improvements in right ventricular metabolism.

Nonstandard Abbreviations and Acronyms

| | |
|------------|---------------------------------|
| FAC | fractional area change |
| FDR | false discovery rate |
| PAH | pulmonary arterial hypertension |

findings: patients with PAH have an elevated prevalence of insulin resistance and impaired fatty acid metabolism compared with matched controls.^{10–14} Noninvasive imaging studies demonstrate increased glycolysis in the human PAH RV,¹⁵ and we have reported evidence of lipotoxic RV triglyceride accumulation in PAH RV.^{7,11,16} There is currently no cure for PAH and the availability of Food and Drug Administration-approved, safe, and well-tolerated metabolism-targeted therapies coupled with the high clinical relevance of metabolic disease in PAH makes study of metabolic interventions in this disease potentially relevant.

There are no approved therapies for right heart failure in patients with PAH despite its high mortality. Multiple lines of evidence demonstrate that impaired glucose and lipid homeostasis affect the RV in PAH;^{1,9,11,14,16–18} however, whether correction of these alterations in human PAH is beneficial or may specifically affect the RV is largely unexplored. Metformin therapy in a rodent model of PAH reduces myocardial lipid and improves some indices of RV function.⁷ The mechanisms through which metformin may work are multiple, including improved insulin sensitivity, enhanced fatty acid oxidation, and reduced oxidant

stress.¹⁹ Metformin is generally well tolerated with an excellent safety profile in humans including those without manifest diabetes mellitus,²⁰ making it a potentially effective metabolic therapy in human PAH that may reduce oxidant stress. Further, left ventricular myocardial triglyceride is known to be increased in type 2 diabetes mellitus^{21–23} and can be modified by caloric restriction,²⁴ suggesting that right ventricular lipid may also be modifiable.

We sought to test the hypothesis that metformin therapy is safe in human PAH and that it may have beneficial effects on the RV via exploratory analyses. We performed an open-label, single-arm phase II study to examine the safety and potential efficacy of metformin in patients with PAH. Our secondary exploratory end points were selected to assess potential mechanisms through which metformin may affect RV function and lipid content using proton magnetic resonance spectroscopy. Further, we explored the metabolomic consequences of metformin therapy in PAH to define potentially important pathways altered by this therapy and identify patients most likely to respond to therapy.

METHODS

The authors will make the data, analytic methods, and study materials reported here upon request in accordance with our institutional review board and Vanderbilt University Medical Center policies. We performed a single-center, open-label, single-arm study of the safety and efficacy of metformin in patients with PAH. The study was approved by Vanderbilt's institutional review board and all subjects gave written informed consent. The study was registered on clinicaltrials.gov before the first subject was enrolled (NCT01884051). Full details of the protocol can be found in Data S1. The primary safety outcomes were development of clinically significant lactic acidosis and study withdrawal attributed to metformin. The primary efficacy outcome was change in plasma markers of oxidant stress (isoprostanes, isofurans). Secondary outcomes included change in measures of glucose and lipid homeostasis, RV function on echocardiography, 6-minute walk distance, myocardial lipid content (in subjects without a contraindication to cardiac magnetic resonance imaging), brain natriuretic peptide levels, and World Health Organization functional class. In exploratory analyses, we examined changes in metabolomic profiles before and after exposure to metformin. Details of testing procedures and biochemical assays are provided in Data S2. Safety was monitored by an independent data safety monitor (TR).

Inclusion and Exclusion Criteria

Full inclusion and exclusion criteria are presented in Data S1. Briefly, we included subjects who were ≥ 18 years of age diagnosed with heritable or idiopathic PAH. Relevant exclusion criteria included known type 1 diabetes mellitus, prednisone use, and evidence of chronic kidney disease or cirrhosis.

Study Procedures

After providing informed consent, subjects underwent testing at baseline and again after 8 weeks. Baseline testing included phlebotomy, 6-minute walk distance, transthoracic echocardiography, and proton magnetic resonance spectroscopy to quantify myocardial triglyceride in a subset. Details of study procedures are described in Table S1. Interim visits at weeks 2, 4, and 6 for safety labs were conducted for the first 12 subjects. After no safety concerns emerged, the interim visits during weeks 2 and 6 were replaced with phone calls for the subsequent 8 subjects. All subjects received phone calls at 2, 4, and 6 weeks. Metformin was given in escalating doses as follows: 500 mg daily for 1 week, 500 mg twice daily for 1 week, 500 mg three times daily for 1 week, and 1 g twice daily for 5 weeks.

Statistical Analysis

We used standard graphing and screening techniques to detect outliers and to ensure data accuracy. We reported summary statistics for both numerical (mean \pm SD) and categorical (proportion and count) baseline demographics. For a comprehensive set of safety end points, insulin-related end points, echocardiogram data, and 6-minute walk distance, we reported summary statistics (mean \pm SD) at baseline and at the end of the study (week 8). Their change from baseline was evaluated using paired *t* test. The data analysis was conducted using the open-source statistical package R (R Core Team, 2017)²⁵.

For the responder analysis, metabolomic data were scaled to set the median level of each metabolite to 1 across all samples. The ratio of postmetformin levels and premetformin levels was obtained for each patient and metabolite and compared to the difference between postmetformin and premetformin triglyceride content by magnetic resonance imaging. Patients were defined as high responders to metformin if their triglyceride content was decreased by 50% or greater after treatment, whereas low responders all had a $< 50\%$ decrease in content. The ratio of post- and premetformin levels of each metabolite were then compared between responders and nonresponders by Mann-Whitney nonparametric test, and $P < 0.05$ was considered significant because the small sample size

and exploratory nature of the analysis. Treating metabolite measure as count data, R package “DESeq2” was used to assess posttreatment metabolomic changes from baseline. In this analysis each metabolite was modeled using a Negative Binomial distribution. The posttreatment metabolite count was analyzed using generalized linear model with baseline in the model and the posttreatment log₂ fold change was tested using the Wald test. Separate analyses were conducted to generate results with and without adjusting for fractional area change. False discovery rate (FDR) adjusted *P* values were reported. A cluster analysis was performed using metabolites with an FDR-adjusted $P < 0.1$. Data were first scaled and then log-transformed to improve the distribution. Results were graphically presented with heatmaps. The linear relationship between pre- versus posttreatment change in metabolite level and the fractional area change (FAC) was assessed using Spearman’s correlation coefficients. Linear mixed-effect models were fitted to estimate the association between metabolite level and FAC. The fixed effect factors are treatment and time point (pre and post). Subjects were treated as a random effect factor to take into account the correlation between pre- and posttreatment measurements.

Metabolomics pathway analysis was performed using the R package “FELLA,” a tool for building hierarchical network representation of the chosen organism using the KEGG database. Metabolites satisfy the following conditions were used in the pathway analysis: (1) FDR-adjusted $P < 0.1$, (2) $< 25\%$ missing data, (3) not in the Xenobiotics pathway, and (4) with a KEGG ID. Thirty-three and 40 metabolites were used as input for the pathway analysis, respectively for analysis adjusted and not adjusted for FAC. Diffusion *P* scores based on the heat diffusion model were generated to compare a node to its null distribution under input permutation. The idea behind the heat diffusion is the usage of the finite difference formulation of the heat equation to propagate labels from the metabolites to the rest of the graph. The top scoring nodes contain not only relevant pathways, but also the intermediate entities that build a plausible explanation on how the input metabolites translate into reported pathways. For visualization purposes, top 100 prioritized by *P* score were displayed on the graphs. The hypergeometric test was used to assess whether a biological pathway contains more hits within the input list than expected from chance given its size. Pathways were ranked according to their *P* value after multiple testing correction. The multiple testing was adjusted using the Benjamini-Hochberg FDR criteria.²⁶ Such adjusted *P* values were then compared against a prespecified FDR of 0.05 as they are reported as adjusted $P < 0.05$. The same adjustment was applied to the metabolomics

analysis. For other analyses, the nominal *P* value was reported.²⁶

RESULTS

Study Population

We screened 68 patients (see Figure 1) and enrolled 20 subjects (85% female) with an age of 42 ± 10 years and body mass index of 30 ± 6 kg/m² (Figure S1). All consented subjects completed the study. Subject demographics and baseline clinical features are shown in Table 1. Most subjects had idiopathic PAH and 8/20 (40%) were receiving intravenous prostacyclin therapy. Mean 6-minute walk distance in our cohort was 434 ± 75 m. Features of the metabolic syndrome²⁷ were common: 3 or more features of the metabolic syndrome were present in 5 patients.

Primary End Point: Safety

A total of 19/20 (95%) subjects reached the target metformin dose, and 1 subject was able to tolerate only a maximal dose of 1500 mg per day. The most common side effects were diarrhea, which occurred in 9/20 (45%) subjects, followed by heartburn (20%) and abdominal pain (15%) (Table 2). There were no serious adverse events and all subjects completed the 8-week protocol. No subjects experienced clinical signs or symptoms of lactic acidosis. No patient required adjustment of PAH therapies or diuretics during treatment with metformin for worsening symptoms. There was no significant change in functional class (*P*=0.99). We

observed a 1.9 kg reduction in body weight, consistent with known effects of metformin.

Primary End Point: Biochemical Outcomes

Although it was not significant, we observed a trend to reduction in the primary efficacy end point of plasma isoprostane (0.041 ± 0.017 ng/mL pre versus 0.035 ± 0.019 ng/mL post, *P*=0.18, Figure S2A). Similarly, the ratio of isoprostane/isofurans did not change significantly (8.5 ± 5.7 pre versus 8.3 ± 5.4 post, *P*=0.98, Figure S2B), though plasma isofurans were commonly undetectable. There was a trend to reduction in glycosylated hemoglobin from $5.33 \pm 0.42\%$ to $5.21 \pm 0.27\%$ (*P*=0.07) and there was no significant change in fasting insulin, glucose, or Homeostatic Model Assessment of Insulin Resistance (pre 1.9 ± 1.7 versus post 1.7 ± 1.0 , *P*=0.57, Table S2). There was no effect on plasma lipid profile (Table S2).

Exploratory End Points: Clinical, Echocardiography, and RV Lipid Content

Metformin had no effect on 6-minute walk distance in our study (pre 434 ± 75 versus post 424 ± 68 m, *P*=0.26) or brain natriuretic peptide level (66 ± 77 pg/mL versus 80 ± 159 pg/mL, *P*=0.60).

Full echocardiographic data are shown in Table 3 before and after metformin exposure. We observed a 3% absolute increase in FAC, a global measure of RV systolic function (pre $23 \pm 8\%$ versus post $26 \pm 6\%$, *P*=0.02, Table 3, Figure S3). There were no changes in measures of RV longitudinal function (tricuspid annular

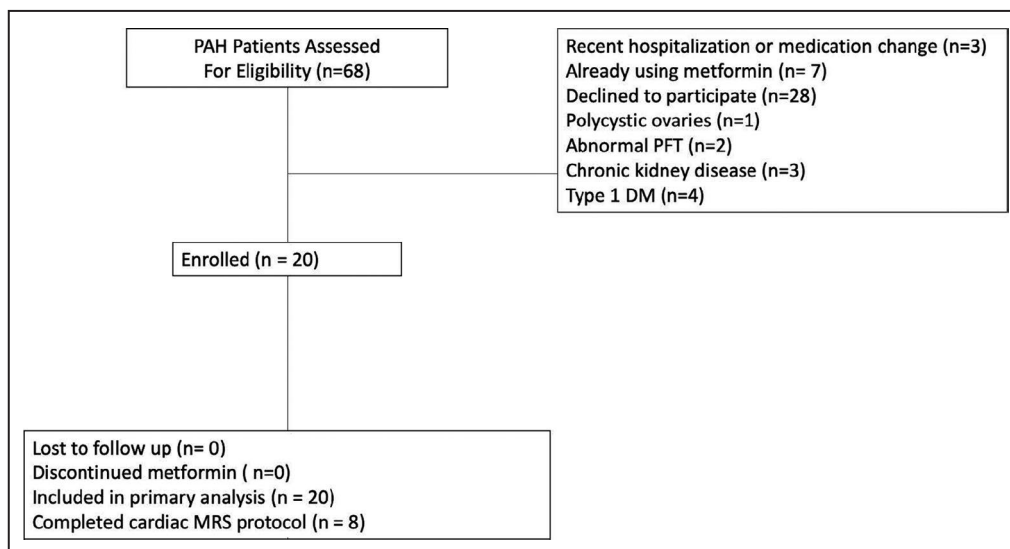


Figure 1. Flow diagram.

DM indicates diabetes mellitus; MRS, magnetic resonance spectroscopy; PAH, pulmonary arterial hypertension; and PFT, pulmonary function test.

Table 1. Baseline Characteristics

| Variable | Value |
|---|-----------|
| Age, y | 42±10 |
| Sex, n (%) female | 17 (85) |
| Race, n (%) | |
| White | 17 (85) |
| Black | 3 (15) |
| Body mass index, kg/m ² | 30±6 |
| PAH etiology (heritable PAH/idiopathic PAH) | 4/16 |
| PAH medications, n (%) | |
| Phosphodiesterase type 5 inhibitor | 13 (65) |
| Endothelin receptor antagonist | 5 (25) |
| Intravenous/subcutaneous epoprostenol or treprostinil | 10 (50) |
| Inhaled prostacyclin analog | 2 (10) |
| >1 PAH therapy | 12 (60) |
| 6-minute walk distance, m | 434±75 |
| World Health Organization functional class | |
| I | 8 |
| II | 12 |
| Features of metabolic syndrome*, n (%) | |
| Elevated glucose | 3 (15) |
| HDL cholesterol | 6 (30) |
| Triglycerides | 9 (45) |
| Obesity | 10 (50) |
| Systemic hypertension | 3 (15) |
| Number of MS features, n (%) | |
| 0 | 4 |
| 1 | 7 |
| 2 | 4 |
| 3 | 4 |
| 4 | 1 |
| Right heart catheterization, most recent | |
| Right atrial pressure, mm Hg | 8.1±2.5 |
| Mean pulmonary artery pressure, mm Hg | 45.5±14.0 |
| Pulmonary artery wedge pressure, mm Hg | 13.0±5.1 |
| Cardiac index, L/min per m ² | 2.4±0.7 |
| Pulmonary vascular resistance, Wood units | 7.3±4.1 |

Data are presented as mean±SD. HDL-C indicates high-density lipoprotein cholesterol; PAH, pulmonary arterial hypertension; MS, metabolic syndrome.

*National Cholesterol Education Program Adult Treatment Panel III criteria used²⁷: glucose > or =100 mg/dL, HDL cholesterol <40 mg/dL men or <50 mg/dL women or drug treatment for low HDL-C, triglycerides > or =150 mg/dL or drug treatment for elevated triglycerides, obesity waist > or =102 cm men, > or =88 cm women, hypertension > or =130/85 or drug treatment for hypertension.

plane systolic excursion, S'; Table 3). There was no change in tricuspid regurgitant velocity or estimated pulmonary artery systolic pressure.

Eight subjects had no contraindication to cardiac magnetic resonance imaging and were able to complete the imaging protocol. Myocardial triglyceride was markedly elevated at baseline^{28,29} and decreased

Table 2. Subject Reported New Side Effects and Adverse Events

| Symptom | Frequency |
|----------------------|-----------|
| Nausea | 2 (10%) |
| Diarrhea | 9 (45%) |
| Vomiting | 1 (5%) |
| Abdominal pain | 3 (15%) |
| Blue lips or fingers | 1 (5%) |
| Chest pain | 1 (5%) |
| Dyspnea | 3 (15%) |
| Edema | 3 (15%) |
| Fatigue | 2 (10%) |
| Syncope | 0 |
| Heartburn | 4 (20%) |
| Dizziness | 0 |

Data are presented as N (%).

significantly with metformin (3.2±1.8% to 1.6±1.4%, $P=0.015$; Figure 2). Triglyceride content declined in 8/9 subjects.

Exploratory End Point: Metabolomics

In an exploratory analysis, we sought to define peripheral blood correlates with significant improvement in RV triglyceride content, defining high responders as a ≥50% reduction in RV triglyceride content between initial and follow-up magnetic resonance spectroscopy. Using non-targeted metabolomics (Metabolon, Research Triangle Park, NC), we found significant differences in metabolites of potential relevance to pulmonary hypertension and right heart failure including dihydroxybutyrate, acetylputriscene, hydroxystearate, and glucuronate (Figure 2B, all adjusted $P<0.05$ versus baseline, see Table S3 for full list of significant metabolites).

Table 3. Echocardiographic Measurements Before and After Metformin

| Variable | Baseline | 8 wk | P Value |
|---|----------|----------|---------|
| Left ventricular ejection fraction, % | 70±5 | 70±5 | 0.67 |
| Left atrial volume index, mL/m ² | 21±8 | 20±6 | 0.51 |
| Ratio of early mitral inflow velocity/mitral annular early diastolic velocity | 10.4±3.5 | 10.8±2.7 | 0.60 |
| Right ventricle basal dimension, cm | 4.4±0.6 | 4.4±0.6 | 0.90 |
| Tricuspid regurgitant peak velocity, m/s | 3.3±0.7 | 3.4±0.7 | 0.90 |
| Right ventricle systolic pressure, mm Hg | 51±21 | 52±21 | 0.85 |
| Tricuspid annular plane systolic excursion, cm | 1.9±0.4 | 2.0±0.3 | 0.37 |
| Tricuspid S', mm/s | 11.8±3.5 | 11.5±2.1 | 0.23 |
| Fractional area change, % | 23±8 | 26±6 | 0.02 |

Data are presented as mean±SD.

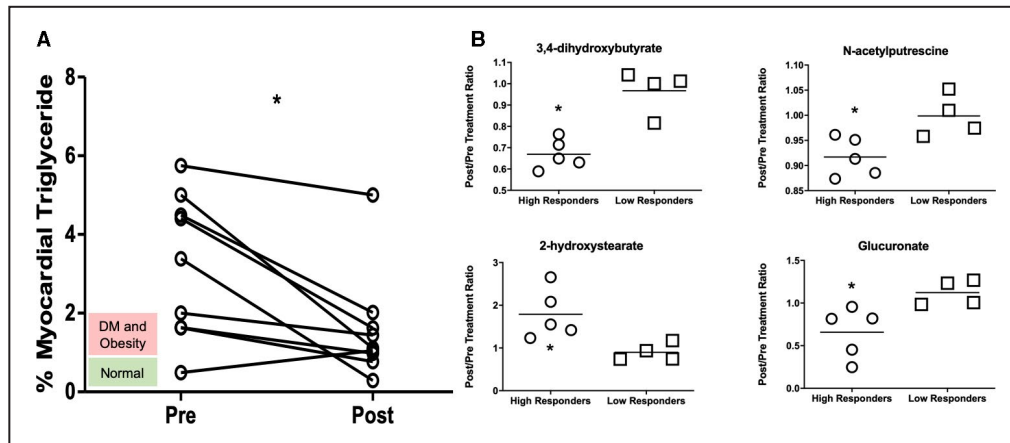


Figure 2. Effect of metformin on right ventricular triglyceride content and metabolomic correlates of improvement.

A, Change in myocardial triglyceride content by cardiac magnetic resonance spectroscopy (n=8). Published reference ranges for obesity and diabetes mellitus are shown.^{28,29} **B**, Plasma metabolomic pattern was compared pre- and postmetformin therapy and segregated by $\geq 50\%$ reduction in right ventricular (RV) triglyceride (Responders) or $< 50\%$ reduction (non-responders). Significantly different ratios were identified in several plasma metabolites of relevance to PAH and lipid and glucose metabolism. * $P < 0.05$.

We further sought to determine which pathways metformin might be affecting including all enrollees. In total, 143 biochemicals were significantly different between baseline and end point with 84 increased and 58 decreasing in concentration. Using a principal clustering analysis, there was not a significant shift between the 2 groups in aggregate; however, when analyzing individual patients, there were noted significant alterations as a function of metformin therapy as shown in Figure S4 and demonstrated in the hierarchical clustering analysis in Figure 3A. A random Forest analysis demonstrated biomarkers that may separate the 2 groups (baseline and after therapy). A predictive accuracy of 80% was identified, which is improved from 50%, which would be expected by random chance. Separation was driven most strongly by lipid metabolites, which made up the top 5 most important to group separation (Figure 3B). Markers of lipid beta-oxidation such as the medium and long-chain acylcarnitine, whose synthesis is an entry point to mitochondrial beta oxidation, were increased after metformin therapy (hydroxybutyrylcarnitine, hydroxypalmitoylcarnitine, and hydroxyoleoylcarnitine all adjusted $P < 0.05$ versus baseline).

In addition to the changes in lipid metabolites with metformin therapy, there were other pathways that were significantly affected. There was a small, nonsignificant decrease in glucose, whereas levels of lactate and pyruvate were increased significantly at end point compared with baseline (1.20- and 1.28-fold increase respectively, adjusted $P < 0.05$). Although there was no clinically significant lactic acidosis, these changes likely reflect an increase in aerobic glycolysis rates with

metformin therapy. Similarly reflecting reduced glucose availability, glucose-related metabolism markers, for example, pentose metabolites, were significantly decreased at end point compared with baseline (adjusted $P < 0.05$). Metformin therapy was associated with significant reductions in metabolites in the urea cycle, tryptophan metabolites, and other markers of inflammation such as DiHOMEs produced by active leukocytes and markers of oxidative stress³⁰ (adjusted $P < 0.05$ for all versus baseline, Figures S5 through S7).

We next sought to understand the changed metabolites using a pathway analysis. Metabolites were included in the analysis if they had (1) FDR-adjusted $P < 0.1$, (2) $< 25\%$ undetectable data, (3) not in the Xenobiotics pathway, and (4) an assigned KEGG ID. For visualization purposes, the top 100 nodes prioritized by P score are displayed in Figure 4. We identified the following key nodes in descending order of strength of association: arginine, malate, 4-methyl-2-oxopentanoate, mannose, citrulline. The sex steroids dhydroepiandrosterone and androstenediol monosulfate were also identified as significantly changed by metformin (1.32- and 1.24-fold higher respectively, adjusted $P < 0.05$).

DISCUSSION

We sought to determine the safety and a potential signal for efficacy for metformin in PAH. Because our prior work in rodent models suggested that metformin improves RV function and lipid deposition in the PAH RV⁷ and prior literature had noted improved oxidant stress

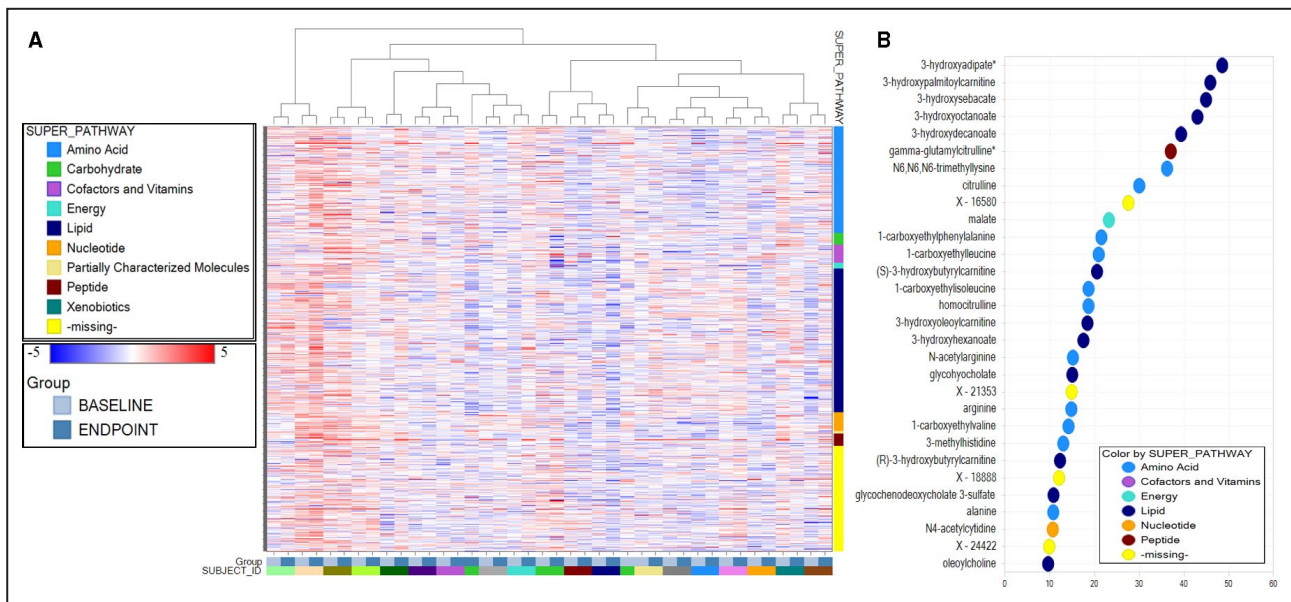


Figure 3. Effects of metformin on plasma metabolome in pulmonary arterial hypertension.

A, Heatmap demonstrating significantly altered pathways after metformin therapy. **B**, Random Forest analysis of baseline vs end point demonstrating significantly altered metabolites and their pathway.

with metformin in human studies and some, but not all, rodent models,^{31–34} we focused on these potential exploratory efficacy metrics in our trial. First, our study demonstrated that metformin is likely well tolerated in PAH. Although there was no significant change in plasma markers of oxidant stress, the trend was toward reduction in these markers and not worsening. Our exploratory analyses further suggested that metformin may improve RV function and reduce RV lipid deposition in PAH.

Most important, metformin appeared to be safe in patients with PAH without advanced heart failure symptoms. We enrolled a high proportion of patients with parenteral prostacyclin therapy (50%) and combination PAH medical therapy (60%), suggesting a patient population with a significant burden of pulmonary vascular disease and currently using drugs with well-described gastrointestinal side effects including diarrhea.^{35,36} Despite this, only 1/20 patients did not reach the target dose owing to side effects. Further, there are reports of metformin causing lactic acidosis in the literature; however, the incidence is so rare that metformin is now considered safe in patients with heart failure.^{19,37} We thus thought it was safe to proceed with a trial in a disease categorized by right heart failure. Most enrolled patients had evidence of reduced RV FAC and, yet, tolerated the drug well. Although there was a nonclinically significant increase in plasma lactate by metabolomics, this biochemical alteration may reflect the metabolic effect of metformin on lactate metabolism,^{31,32} rather than a clinically important lactic acidosis as no patient suffered an adverse event.

Our prior work had demonstrated in an animal model of RV failure in PAH that metformin improves RV function and may improve histologic evidence of lipid deposition.¹¹ Further, we have previously published that patients with PAH had increased RV triglyceride content.¹¹ Based on these data, we tested the hypothesis that metformin therapy improves RV lipid content and function in human patients with PAH. We tested this hypothesis using echocardiography, because of its clinical relevance. In addition, we recruited a subcohort of patients to undergo cardiac magnetic resonance spectroscopy. Despite relatively few enrollees, we observed a significant decrease in percentage of cardiac triglyceride, signifying reduced RV lipid content. We did not measure invasive hemodynamics; however, there was no change in RV systolic pressure estimates, suggesting that if there are pulmonary vascular effects of metformin therapy, these are less pronounced than the effects on the RV. Although metformin did not normalize RV FAC, there was a significant improvement even in our short-term study of RV function with an absolute difference of 3% from baseline to study end, which may be clinically significant.³⁸ Taken together, these data suggest that metformin could have a direct effect on the failing RV and that metformin's RV effects should be tested in a more robust fashion in future studies.

We further hypothesized that peripheral blood metabolomic changes may reflect RV metabolism. We thus sought metabolomic correlates in the peripheral blood of subjects who achieved a >50% reduction in RV lipid content in an exploratory analysis.

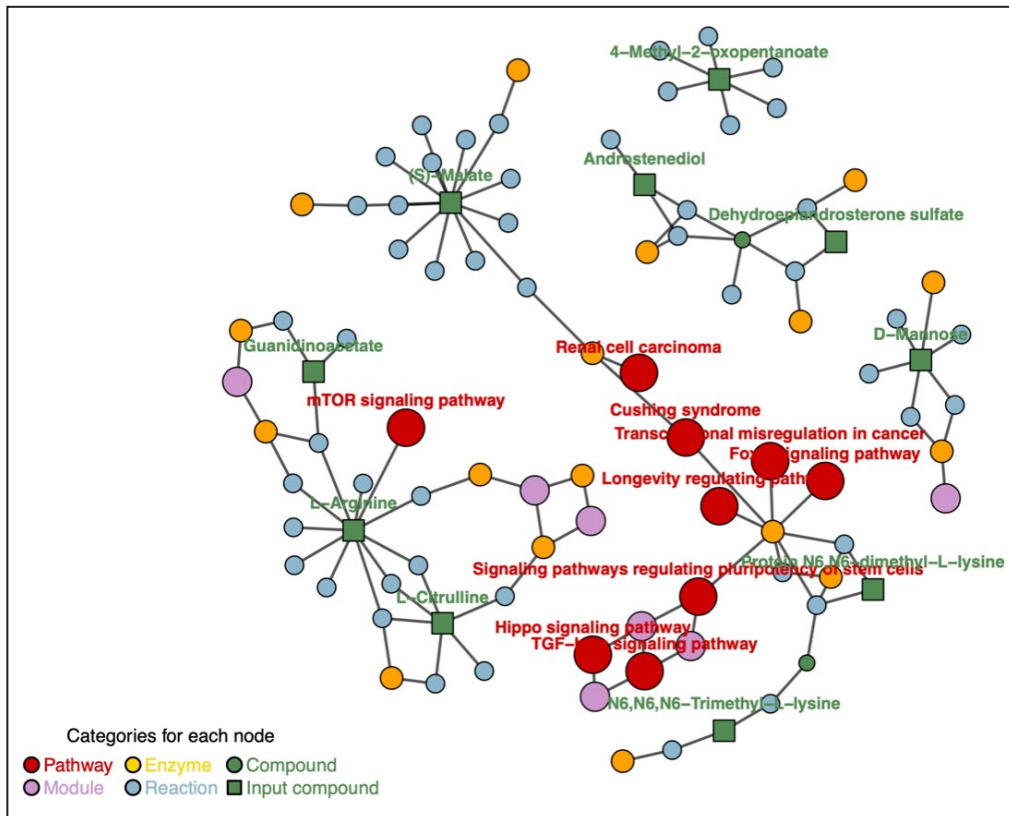


Figure 4. Pathway analysis to identify key nodes in changed metabolites.

We identified several compounds that may be of relevance to RV and PAH metabolism. Dihydroxybutyrate, a ketone synthesized from fatty acids during ketogenesis, was reduced, which may suggest enhanced lipid beta oxidation. Elevation of this metabolite has been reported in children with persistent pulmonary hypertension of the newborn indicated potential relevance to pulmonary vascular disease.³⁹ Acetylputrescine was elevated and associated with mortality in a large multinational cohort of patients with PAH⁴⁰ and was reduced in patients with significant reduction in RV triglyceride after metformin in our cohort. Alterations in hydroxysterate and glucuronate similarly suggest potential changes in lipid and glucose metabolism of relevance to PAH.^{41,42} Safai et al recently demonstrated the effect of metformin on branched chain amino acid metabolites in a clinical trial,⁴³ however, the larger, particularly extended lipid metabolite, effects of metformin in humans are largely unknown. Our pathway analysis suggests a major effect of metformin on urea cycle metabolism, sex hormones, and glucose and fat metabolism in patients with PAH. Although our data do not show that RV lipid deposition accelerates RV failure, or that these biomarkers reflect RV composition changes specifically, they do suggest there may potentially be peripheral blood markers of drug responses specific

to metformin and to the RV that could be studied prospectively.

Our work has limitations. This was a single-center, open-label study focused on safety and potential early markers of efficacy and thus there was no control group and we were underpowered to detect markers of clinical efficacy. Our RV findings, in particular, require confirmation in a larger, multicenter trial that is now enrolling (NCT 03617458). We were unable to measure RV ejection fraction by magnetic resonance imaging, a more accurate measure of RV function than echocardiographic metrics, due to the duration of the spectroscopy protocol and we recognize that the low number of participants in this procedure may bias to error, however the results were nonetheless compelling and the body mass index was similar in this substudy (30±4.8) to the rest of the trial participants. We did not identify a significant change in plasma markers of oxidant stress (isoprostanes, isofurans); however, we also did not find any concerning worsening of these markers either that would preclude further study. Although approximately half of our patients were obese,⁴⁴ they are similar in body mass index to other US-based studies of PAH.^{9,45} The positive exploratory analyses are likely not attributable to simply improving obesity as the final mean body mass index was

29.7±5.9. Additionally, although all plasma samples were taken in the fasting state and subjects were not instructed to change their diet, we cannot rule out a change in diet that is reflected in the plasma metabolome.

In conclusion, metformin therapy was safe and well tolerated in patients with PAH in this single-arm, open-label mechanistic phase II study. Exploratory analyses suggest that metformin may be associated with improved RV FAC and, in a subset of patients, reduced RV triglyceride content that correlated with altered lipid and glucose metabolism markers. Further study of metformin in PAH is warranted.

ARTICLE INFORMATION

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Supplementary Material

Data S1–S2

Tables S1–S3

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Supplemental Material

Data S1.

Supplemental Methods

The protocol specified recruitment of 27 subjects. Enrollment was stopped after recruitment of 20 subjects due to termination of the funding source. The first subject was recruited in April 17, 2015 and the final subject completed the protocol in March 28, 2018.

Inclusion criteria:

1. Over 18 years old, of either sex and any race
2. Known diagnosis of heritable or idiopathic PAH, WHO functional class I-III

Exclusion criteria:

1. Pregnancy
2. Type 1 DM or polycystic ovarian disease
3. Prednisone use
4. PAH other than idiopathic or heritable
5. FVC<70% or FEV1 < 60% predicted
6. Creatinine > 1.5mg/dL
7. Contraindication to Cardiac MRI
8. Liver transaminases (AST, ALT) > 2x the upper limit of normal for Vanderbilt's clinical laboratory
9. Inability to provide informed consent

10. No evidence of more than mild right heart failure on physical examination
11. Hospitalization or PAH medication change within 3 months

Study Procedures

After providing informed consent, subjects underwent testing at baseline and again after 8 weeks. Baseline testing included phlebotomy, six minute walk distance, transthoracic echocardiography, and proton magnetic resonance spectroscopy to quantify myocardial triglyceride in a subset. Details of study procedures are described in **Table S1**. Interim visits at weeks 2, 4, and 6 for safety labs were conducted for the first 12 subjects. After no safety concerns emerged, the interim visits during weeks 2 and 6 were replaced with phone calls for the subsequent 8 subjects. All subjects received phone calls at 2, 4, and 6 weeks. Metformin was given in escalating doses as follows: 500mg daily for 1 week, 500mg twice daily for 1 week, 500mg three times daily for 1 week, 1g twice daily for 5 weeks.

Proton Magnetic Resonance Spectroscopy: All image data was acquired on a Philips 3.0T Achieva magnet with R5.1.7 software release. Subjects were placed head first supine inside the 16ch Torso XL coil. All breath hold scans were acquired end expiration while spectra data was respiratory gated and cardiac triggered. Water spectra (suppressed and unsuppressed) was processed using Philips MR software (Spectroview). Unsuppressed spectra data was processed without residual water suppression while suppressed spectra was processed with residual water subtraction. This protocol has been previously published (Brittain et al, Circulation, 2016)

Isoprotane and Isofuran Measurement: Isofurans and Isoprostanes were measured in EDTA plasma by the Eicosanoid Core Laboratory at Vanderbilt University Medical Center.

Metabolomic Analysis: Fasting EDTA plasma was used for metabolomics assays through Metabolon (Research Triangle Park, NC) using both the standard platform and complex lipid platform.

Statistical Analysis:

We used standard graphing and screening techniques to detect outliers and to ensure data accuracy. We reported summary statistics for both numerical (mean \pm SD) and categorical (proportion and count) baseline demographics. For a comprehensive set of safety endpoints, insulin-related endpoints, echocardiogram data, 6 minute walk distance, we reported summary statistics (mean \pm SD) at baseline and at the end of the study (week 8). Their change from baseline was evaluated using paired-t test. The data analysis was conducted using the open-source statistical package R (R Core Team, 2017).

For the responder analysis, metabolomic data were scaled to set the median level of each metabolite to 1 across all samples. The ratio of post-metformin levels and pre-metformin levels was obtained for each patient and metabolite and compared to the difference between post-metformin and pre-metformin triglyceride content by MRI.

Patients were defined as high responders to metformin if their triglyceride content was decreased by 50% or greater after treatment, whereas low responders all had a less

than 50% decrease in content. The ratio of post- and pre-metformin levels of each metabolite were then compared between responders and non-responders by Mann Whitney non-parametric test, and $p < 0.05$ was considered significant due to the small sample size and exploratory nature of the analysis. R package “DESeq2” was used to assess post-treatment metabolomic changes from baseline. Separate analyses were conducted to generate results with and without adjusting for fractional area change. False discovery rate (FDR) adjusted p-values were reported. A cluster analysis was performed using metabolites with an FDR-adjusted p-value < 0.1 . Data were first scaled and then log-transformed to improve the distribution. Results were graphically presented with heatmaps. The linear relationship between pre- vs. post-treatment change in metabolite level and the fractional area change was assessed using Spearman’s correlation coefficients. Linear mixed-effect models were fitted to estimate the association between metabolite level and FAC. The fixed effect factors are treatment and time point (pre and post). Subjects were treated as a random effect factor to take into account the correlation between pre- and post-treatment measurements. Metabolomics pathway analysis was performed using the R package “FELLA”, a tool for building hierarchical network representation of the chosen organism using the KEGG database. Metabolites satisfy the following conditions were used in the pathway analysis: (1) FDR-adjusted p-value < 0.1 , (2) less than 25% missing data, (3) not in the Xenobiotics pathway, and (4) with a KEGG ID. Thirty-three and 40 metabolites were used as input for the pathway analysis, respectively for analysis adjusted and not adjusted for FAC. Diffusion p-scores based on the heat diffusion model were generated to compare a node to its null distribution under input permutation. The idea behind the

heat diffusion is the usage of the finite difference formulation of the heat equation to propagate labels from the metabolites to the rest of the graph. The top scoring nodes contain not only relevant pathways, but also the intermediate entities that build a plausible explanation on how the input metabolites translate into reported pathways. For visualization purposes, top 100 nodes prioritized by p-score were displayed on the graphs. The hypergeometric test was used to assess whether a biological pathway contains more hits within the input list than expected from chance given its size. Pathways were ranked according to their p-value after multiple testing correction.

Data S2.

Vanderbilt Pulmonary Circulation Center Detailed Protocol:

Clinical Trial of Metformin in Pulmonary Arterial Hypertension

I. Background and Significance

A. Historical background and preliminary data

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by progressive obliteration of the pulmonary vasculature, right ventricular (RV) failure and death¹. Despite major advances in understanding development of PAH in recent decades, safe, effective and tolerable therapies remain elusive. Similarly, although RV failure is the predominant cause of death in PAH, no RV-specific therapies are current available. Recently, our group and others have explored the role of the metabolic dysregulation (obesity, insulin resistance, and dyslipidemia) as a pathogenic mechanism in PAH²⁻⁶. We have studied two separate sequela of metabolic dysregulation in humans and experimental models of PAH: systemic and pulmonary vascular oxidant stress and alteration of RV metabolism. Our preliminary data suggest that metabolic modification may represent a new therapeutic avenue in PAH with beneficial effects on both oxidant stress and RV metabolism.

Reactive oxygen species are powerful mediators in both experimental models of pulmonary hypertension and human disease⁷⁻¹⁰. Increased oxidant production and decreased clearance of reactive oxygen species have been described in PAH, resulting in alterations in mitochondrial number and function. Abnormal mitochondrial function results in a higher glycolytic rate in IPAH endothelial cells as indicated by increased glucose utilization on positron emission tomography (PET) scanning of both the pulmonary vasculature and the RV^{11,12}. In addition to ROS production, mitochondrial dysfunction leads to impaired fatty acid metabolism, the preferred source of fuel for the heart¹³. Similar defects in fatty acid metabolism in diseases of the left heart and diabetic cardiomyopathy result in toxic lipid deposition. Our data suggest that similar mechanisms contribute to RV failure in PAH. Evidence in the literature and our preliminary data show that ROS generation and altered glucose and lipid metabolism are hallmarks of PAH in rodent models and human disease. These changes in glucose and lipid metabolism can be quantified and monitored serially with PET, magnetic resonance spectroscopy (MRS) and measurement of isoprostanes and isofurans in human PAH patients.

B. Preliminary Data

Our group has experience examining the interplay between insulin resistance, oxidant stress, and myocardial metabolic dysregulation in PAH. We have shown that insulin resistance is highly prevalent in humans with PAH (independent of body mass index) and is a key feature of our transgenic mouse model of pulmonary hypertension with a mutation in bone morphogenetic protein receptor type II (known to cause heritable and occasionally idiopathic PAH)^{2,3}. Markers of oxidant stress (isoprostanes and isofurans) are elevated in lungs of both humans and mice with PAH and in urine samples in humans with PAH¹⁴.

Metabolic dysregulation is evident in the myocardium in PAH and contributes to RV failure.

We have shown that PAH patients with diabetes have a worse prognosis than non-diabetic PAH patients and the presence of diabetes is associated with impaired RV function. Gene expression arrays of the RV myocardium in 2 patients with PAH who died of RV failure demonstrate increased glycolysis and decreased fatty acid oxidation (FAO) compared to both healthy controls and patients who died of left heart failure¹³. This altered metabolic phenotype led us to discover marked accumulation of triglyceride within the myocardium in our mouse model of PAH and in explanted hearts from humans with PAH. We translated these findings to living patients with PAH by developing a MRS technique to quantify myocardial triglyceride content *in vivo*. Myocardial triglyceride content is 10-30 fold higher in PAH patients compared to healthy controls (Figure X).

Metabolic Imaging

PET imaging is able to quantify the uptake and breakdown of energy substrate metabolites and is therefore well-suited to study metabolic abnormalities in the myocardium. FDG-PET measures the amount of glucose taken up by the heart to undergo glycolysis or form glycogen. Myocardial oxidative metabolism can be measured by PET using the decay rate of carbon 11-labeled acetate (¹¹C acetate). The rate of tissue clearance of ¹¹C acetate (k_{mono}) is tightly coupled to the rate of oxidative phosphorylation in the mitochondria and is thus a reliable surrogate for oxygen consumption and FAO activity in the myocardium¹⁵. It is important to relate the oxidative metabolic rate (energy supply) to the energy demand in the organ of interest. RV metabolic demand can be assessed by the

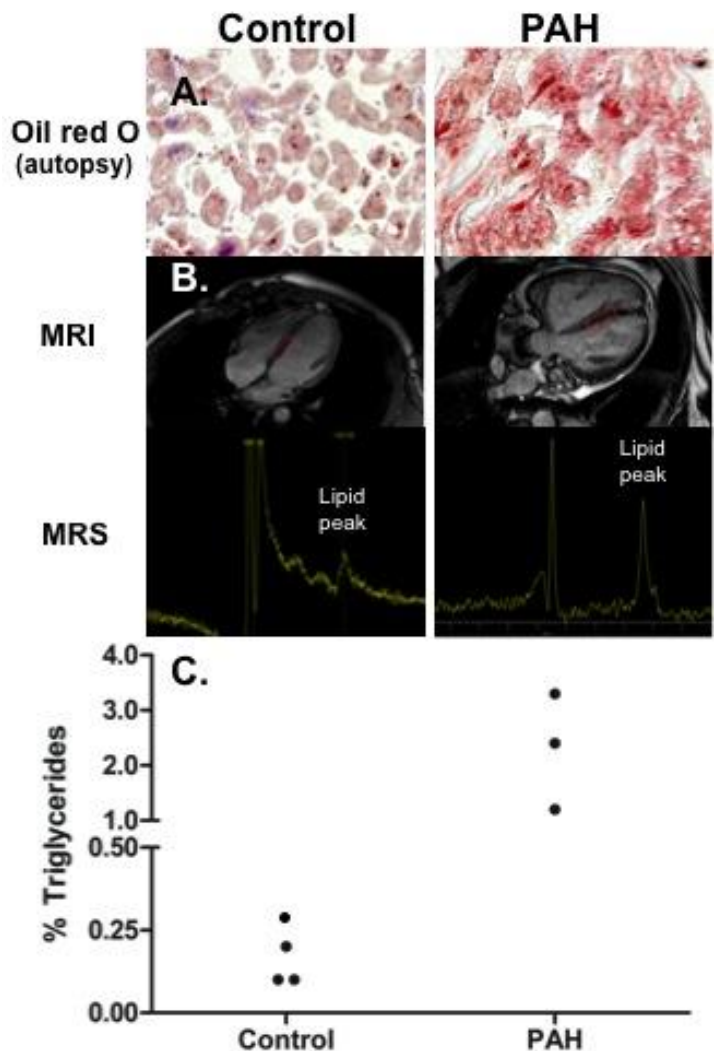


Figure X. (A) Human PAH is associated with extensive oil red O staining (red droplets within cardiomyocytes). Lipid is scant in the disease control human RV with non-ischemic cardiomyopathy. (B) Cardiac MRI and proton magnetic resonance spectroscopy (MRS) of normal subject demonstrates normal RV function and small lipid peak. PAH patient has RV dilation and hypertrophy with prominent lipid peak. (C) Quantified intra-myocyte lipid content shows markedly elevated triglyceride in PAH compared to normal subjects. Intra-patient reproducibility within $\pm 0.05\%$.

rate pressure product (heart rate x pressure; RPP) using the estimated RV systolic pressure from Doppler echocardiography.

Metformin in PAH Metformin is an orally administered biguanide used in the treatment of insulin resistance found in type 2 DM and polycystic ovarian disease¹⁶. Metformin acts by enhancing insulin sensitivity, inducing higher levels of peripheral insulin uptake and decreasing hepatic gluconeogenesis. A major effect of this drug is inhibition of the enzymatic activity of complex I of the mitochondrial respiratory chain, the primary generation site for reactive oxidant species including superoxide¹⁷⁻¹⁹. Moreover, data in cardiac myocytes show that metformin attenuates mitochondrial dysfunction in heart failure through activation of adenosine monophosphate-activated kinase²⁰. Metformin improves LV function and hemodynamics in human LV failure and decreases lipid deposition in animal models of LV failure but has not been tested in the RV²¹⁻²³. Meta-analyses of controlled trials of anti-diabetic agents in heart failure have shown that metformin significantly reduces mortality and hospitalization rate compared with other agents, despite similar effects on HgbA1C levels. Metformin is now considered safe in heart failure^{24,25}. Metformin is also used in polycystic ovarian disease without hyperglycemia, showing safety in the non-hyperglycemic population¹⁶. Thus metformin has several potential effects in PAH that would test our molecular hypothesis: Specifically, improving insulin resistance would improve glucose control in hyperglycemic patients, improve mitochondrial function with lower ROS production, offer potential cardioprotection, decrease lipotoxicity, and improve respiratory coupling with subsequent reduced proliferative effects. In a rodent monocrotaline PAH model, metformin also showed a protective effect²⁶, but there have been no trials of this drug in human PAH. We have 10 patients in our clinic who have used metformin as an anti-diabetic and not experienced any adverse effects. We plan to use metformin as an agent to test our hypothesis that treatment of insulin resistance in patients reduces oxidant stress, ameliorates adipokines, improves six minute walk distance and, potentially, clinical outcomes.

In this proposal, we will test our central hypothesis that metformin therapy will reduce oxidant stress and improve myocardial energetics in humans with PAH.

C. Rationale and Potential Benefits

Our preliminary data in humans with PAH and experimental models point to a role for insulin resistance and development of oxidant stress in the pulmonary vasculature and altered myocardial metabolism in the RV. These associations are important to explore for several reasons. First, metabolic syndrome and its individual components, particularly insulin resistance, are on the rise in this country and if features of the metabolic syndrome promote pulmonary vascular disease, this has implications for the incidence of PAH. Second, there are currently available, safe and well tolerated treatments for insulin resistance and

other components of the metabolic syndrome that could potentially be used in PAH. The findings of this study will be directly relevant to a) understanding the role of metabolic modulation in PAH and b) identifying a potentially novel treatment for patients with PAH.

II. Hypotheses and Specific aims

We hypothesize that metformin therapy will reduce oxidant stress and improve myocardial energetics in humans with PAH.

Specific Aim 1. **To test the hypothesis that metformin will ameliorate oxidant stress in pulmonary arterial hypertension**

Primary safety endpoint: absence of lactic acidosis, withdrawal from the study if attributed to metformin

Primary efficacy endpoint: change in urinary and plasma oxidant stress measures (F2 isoprostanes and metabolites, isofurans, and nitrotyrosine)

Secondary Endpoints: lung cellular proliferation as measured by FDG avidity, change in the markers of insulin resistance and sensitivity, BMPR2 expression in peripheral blood mononuclear cells, and change in glucose and lipid metabolites.

Specific Aim 2. **To test the hypothesis that metformin will decrease myocardial lipid content, increase oxidative metabolism and decrease glucose uptake.**

Primary Endpoints: change in myocardial percent triglycerides (%TGs), k_{mono}/RPP of C^{11} acetate, and uptake of FDG before and after metformin.

Secondary Endpoints: change in RVEF, RV mass index, insulin resistance and sensitivity indices, glucose and lipid metabolites, and six-minute walk distance (6MWD)

III. Subject Selection

Inclusion criteria:

1. Over 18 years old, of either sex and any race
2. Known diagnosis of heritable or idiopathic PAH, WHO functional class I-III
3. Stable PAH therapy

Exclusion criteria:

1. Pregnancy
2. Type 1 DM or polycystic ovarian disease
3. Prednisone use
4. PAH other than idiopathic or heritable
5. $FVC < 70\%$ or $FEV1 < 60\%$ predicted
6. Creatinine $> 1.5\text{mg/dL}$
7. Contraindication to Cardiac MRI
8. Liver transaminases (AST, ALT) $> 2x$ the upper limit of normal for Vanderbilt's clinical laboratory
9. Inability to provide informed consent

10. No evidence of more than mild right heart failure on physical examination

IV. Subject Enrollment

Potential participants will be identified through the Vanderbilt Pulmonary Vascular Disease Clinic. Subjects will be given compensation for transportation (voucher for parking and fuel) at all visits. Research subjects who complete the study will be compensated up to \$500 on the following schedule:

Complete visit 1=\$150

Complete visit 2=additional \$50 (total \$200)

Complete visit 3=additional \$50 (total \$250)

Complete visit 4=additional \$250 (total \$500)

Upon identification by the study staff, the potential participant will receive a complete explanation of the protocol procedures in person or by telephone. During that initial conversation, the potential participant will be screened for inclusion and exclusion criteria. In order to identify contraindications to metformin use that are unknown, unrecognized, or undisclosed, the medical record of all potential subjects, when available, will be reviewed by study staff prior to the first study visit. Potential subjects who wish to participate in the study and meet no exclusion criteria will be mailed or given in person a copy of the study protocol and consent with encouragement for review prior to the first study visit. Any interim questions regarding the protocol will be answered over the phone. After reading the study protocol and consent documents, potential subjects who wish to participate will be invited to attend a first study visit. Because fasting blood work will be obtained at the time of the first visit, the participant will be instructed to fast for 8 hours before the visit. For this reason, all first visits will take place between 8 and 10 in the morning. Participants will be recruited and enrolled in an ongoing manner until the study goal of 27 participants is reached. A flowsheet of the study schedule is provided in **FIGURE 1**.

Informed Consent

At the time of the first study visit, the participant will have had the opportunity to read the informed consent and been given the opportunity to ask questions by telephone. At the start of the first visit, the informed consent will be reviewed in person with the potential subject by approved study personnel in a private, comfortable area. Participants' questions about the protocol will be answered thoroughly, and there is no time limit to this procedure. The consent form clearly states that subjects may end participation at any time without prejudice, and this is emphasized to the subject. In particular, subjects who receive their health care at Vanderbilt University Medical Center will be reassured that their decision to decline participation will in no way affect their medical treatment. If the potential participant agrees, a study staff member will obtain written consent and the individual is enrolled into the study.

As part of the informed consent, participants will be asked whether blood, not used in this study, may be stored for future analysis. If the participant refuses, samples will be used only for the present study and excess samples discarded.

Treatment assignment and randomization

All participants in this study will receive metformin in an unblinded fashion. There will be no placebo.

V. Study Procedures

Scheduled Visits

The schedule of visits and the measures to be obtained at each visit are displayed in **TABLE 1**. At the first study visit, participants will have anthropometric measures taken: weight and height will be measured in order to calculate BMI; and waist and hip circumference will be measured to obtain WHR. Information on age and gender will be obtained. Seated blood pressure and pulse will be checked. A medical history will be obtained by study staff, which includes information on current and past medical issues, family history of illness, medications, and allergies to past medications in order to identify possible study contraindications. Participants meeting exclusion criteria at the study visit will be notified and an explanation provided for why they are excluded from the study.

FIRST STUDY VISIT

For those without exclusions, a drawing IV will be placed so that blood draws can be performed with minimal discomfort. Fasting blood work will be obtained; specifically, measures of glucose, insulin, BNP, NTpro-BNP, hemoglobin A1C, creatinine, and hepatic transaminases (AST and ALT), hematocrit (HCT), and beta-hcg (if female). For these fasting blood samples, 45cc of blood is needed. We will collect an additional 30cc (3 X 10cc vials) and store for additional, future analyses. Only the creatinine, beta-hcg, and hepatic laboratories will be processed immediately in order to screen for study exclusion criteria. The remaining laboratories are stored at -80°C and will be processed as a batch at the end of the study.

If the results of the eGFR and hepatic transaminases are acceptable for study participation and a serum pregnancy test is negative, the subject will then be undergo additional testing on the same day (**FIGURE 1**). If the results of the eGFR or hepatic transaminases meet exclusion criteria, they will be discussed with the patient. In this case, additional study visits will be cancelled, and the subject will be excluded from the protocol. Study staff members are available for questions at any time throughout the study, and contact numbers are provided to the subject.

6MWD We will use the six-minute walk distance to determine subjects functional capacity at base line and any changes that occur over the course of the study

Echocardiography We will use to evaluate change in RVEF, RV mass index, other indices of right ventricular pathology.

Cardiac Steatosis and MR Spectroscopy (MRS) We will use MRS to measure the amount of intra-myocyte triglyceride in PAH patients and controls. MRS will quantify myocardial triglyceride as a percent versus water content, from the formula: [signal amplitude of triglyceride/signal amplitude of water] X 100. The usual components of a cardiac MRI will also be obtained from the MRS scan.

PET Imaging PET imaging is able to quantify the uptake and breakdown of energy substrate metabolites and is therefore well-suited to study metabolic abnormalities in the myocardium. FDG-PET measures the amount of glucose taken up by the heart to undergo glycolysis or form glycogen. Myocardial oxidative metabolism can be measured by PET using the decay rate of carbon 11-labeled acetate (^{11}C acetate).^{45,46} The rate of tissue clearance of ^{11}C acetate (k_{mono}) is tightly coupled to the rate of oxidative phosphorylation in the mitochondria and is thus a reliable surrogate for oxygen consumption and FAO activity in the myocardium. It is important to relate the oxidative metabolic rate (energy supply) to the energy demand in the organ of interest. The balance of supply and demand may be more important to organ function than the absolute metabolic rate.⁴⁷ RV metabolic demand can be assessed by the rate pressure product (heart rate x pressure; RPP) using the estimated RV systolic pressure from Doppler echocardiography.⁴⁸ PET imaging with FDG and ^{11}C acetate is the best available tool to measure the relative activity of glycolysis and FAO in the human myocardium.

At the completion of the first study visit, participants will be given 8 weeks of metformin and detailed instructions for dose escalation according to the protocol. Participants will be instructed to begin treatment on the evening of the first study visit. Participants will be called twice weekly for the first 2 weeks and then weekly thereafter to ensure compliance and assess for tolerance and side effects.

VISITS 2 and 3

At the second and third study visits, an interim medical history will be taken to assure that no contraindications to study participation have developed, and the subject will undergo blood collection (identical to the first visit) and 6MWD testing (**FIGURE 1**). The subject will be called three days before each visit by study staff in order to remind him or her of the appointment. Since fasting blood work will be obtained at all visits, they will be scheduled between 8 and 10 in the morning, and the subject will be reminded not to eat 8 hours before the visits.

VISIT 4

At the fourth study visit, pill counts will be performed to monitor subject adherence. Blood work and measures of anthropometry will be performed identical to the first study visit (**FIGURE 1**). Patients will then undergo additional testing, including 6MWD, echocardiography, PET scans, and MRS/MRI.

At the end of each study visit, subjects will be given the opportunity to ask any questions regarding the study, and there is no time limit to this. As stated above, study staff is always available to answer study questions at any time and contact numbers are provided to the participants.

Medications

Subjects will receive metformin 500mg tablets and instructed to escalate dosing as below:

- 500mg daily for 1 week
- 500mg twice daily for 1 week
- 500mg three times daily for 1 week
- 1g twice daily for 5 weeks

VII. Biostatistical analysis

Outcomes

The primary outcomes for Specific Aim 1 of this study will be change in urinary and plasma F2 isoprostanes, isofurans, and nitrotyrosine. Secondary outcomes for Specific Aim 1 will be changes in lung FDG avidity, markers of insulin resistance (HOMA-IR), BMPR2 expression in peripheral blood mononuclear cells, and glucose and lipid metabolites

The primary outcomes for Specific Aim 2 will be change in percent myocardial triglycerides (%TGs), $k_{\text{mono}}/\text{RPP}$ of C^{11} acetate, and uptake of FDG before and after metformin. Secondary outcomes for Specific Aim 2 will be the change in 6MWD and the change in RVEF, RV mass index, HOMA-IR, glucose and lipid metabolites.

Definition of Analysis Variables

In addition to the above measures, the following calculated variables will be used in the study:

Body mass index (BMI):

$$\text{Weight in kilogram}/(\text{height in meters})^2$$

Homeostatic Model of Insulin Resistance (HOMA-IR):

Fasting glucose (mg/dL) x fasting insulin (μ U/L) / 405

Analysis Plan for Primary Outcomes

Paired values before and after metformin of the primary outcomes for Specific Aims 1 and 2 will be compared using the Wilcoxon signed-rank test or paired t-test depending on normality of the data. Analyses of measurement after metformin treatment using general linear model (GLM) adjusting for baseline measurement, age, and gender will also be conducted. The log or other appropriate data transformation will be applied if normality is violated in the original scale before these GLM analyses.

For measurement obtained at multiple time points, such as 6MWD measured at baseline and weeks 2, 4, and 8, a repeated measures linear mixed effects model will be used for the analysis with time, age and gender as fixed effects and a random subject effect. The within subject correlation over time will be modeled using the autoregressive correlation structure²⁷. Since all patients are on treatment, the main hypothesis is based on a time effect where the null hypothesis is that the mean response profile is constant over time (i.e., flat). This model handles unbalanced data, allowing for missing measurements. Residual analyses and graphing will be done to verify distributional assumptions.

We will accept as significant any test reaching the nominal threshold $P < 0.05$. Due to the exploratory nature of the study, no multiple comparison adjustment will be made.

Analysis Plan for Secondary Outcomes

Secondary outcomes will be analyzed using the same methods as for the primary outcomes. In addition, to determine whether metformin treatment effects on oxidant stress markers and metabolic imaging endpoints correlate, the association between primary and secondary outcomes will be analyzed using the Spearman or Pearson correlation coefficient depending on the normality of the data. These analyses are primarily exploratory.

Power Analysis

A total of 27 subjects will enter this randomized. Even if there is 20% drop-out (expected during research participation), we will have 99% power at $\alpha = 0.05$ to detect a true difference between groups in F2-isoprostane of 0.67 ± 0.3 pg/mL or in percent myocardial triglyceride of 0.5 ± 0.3 . The estimate for between group differences in F2-isoprostane is based on our own preliminary data in the PAH population and controls ($n = 22$ and 4 , respectively). The estimate for change in percent TG is based on differences observed between insulin resistant and non-insulin resistance populations²⁸. This technique has not previously been applied to the PAH population so sample size calculations are inherently empiric.

VI. Risks and Discomfort

A. Study Procedures: Possible Risks and Protocols for Minimization

Venipuncture All specific aims require patients to have blood drawn for research purposes. The risks of drawing blood are uncommon and may include bleeding, minor infection and bruising. Commonly, having blood drawn is painful, and rarely can lead to infection at the site of the blood draw. The amount of blood drawn is small, and represents an exceedingly small percentage of the amount of the total blood volume and will not represent a significant risk to the patient.

PET Scan Specific Aim 2 requires patients to undergo [¹⁸F] avidity and C11 acetate PET scans. Risks to this procedure include the risk of placement of peripheral IV, small radiation exposure and allergic reaction to radiotracer, though this is extraordinarily infrequent. The radiation risk of this procedure is small, similar to other nuclear medicine studies.

Echocardiography Specific Aim 2 requires patients to undergo echocardiography. There are no risks to this ultrasound procedure aside from minor discomfort from placement of the ultrasound probe.

Metformin This drug has been on the market for several decades and is considered first line therapy for diabetes mellitus type 2. Moreover, it has been used extensively in non-hyperglycemic patients with polycystic ovarian disease. Additional studies of the safety of metformin in heart failure have been published. The pertinent risk of metformin is development of lactic acidosis. This risk has been reported primarily in patients with abnormal renal or hepatic function, thus we have chosen to exclude these populations from the study. We plan to measure lactic acid levels at each study visit to monitor for occult lactic acidosis, though it is usually clinically apparent when there are significant levels in the plasma. Initially we will enroll patients who are functional Class 1 or 2 as they are least likely to have occult organ dysfunction. If they exhibit no adverse events, enrollment will be expanded to include Class 3 patients. Additional concerns include hypoglycemia, though this is a rare risk of this drug, and gastrointestinal distress. We will measure fasting glucose at weeks 2 and 4 to assess for fasting hypoglycemia. Patients will be educated on the signs and symptoms of hypoglycemia and advised to contact study staff immediately if any arise. Several other potential side effects listed in the package insert that will be monitored at study visits and through phone conversation if need arises. We will monitor patients twice weekly for the first two weeks through either a visit or phone conversation and once thereafter for the duration of treatment. At these points, side effects that limit dose adjustment will be assessed. If patients report significant gastrointestinal effects, metformin will not be increased as would otherwise occur in the protocol. If symptoms persist further, the dose will be dropped by 500 mg daily to the highest tolerated dose.

The most common adverse reactions experienced are:

>10% of patients: diarrhea (instant release tablet: 12% to 53%; extended release tablet: 10% to 17%), nausea/vomiting (instant release tablet: 7% to 26%; extended release tablet: 7% to 9%), flatulence (12%), Weakness (9%)

1% to 10%: chest discomfort, flushing, palpitation, headache (6%), chills, dizziness, lightheadedness, rash, hypoglycemia, Indigestion (7%), abdominal discomfort (6%), abdominal distention, abnormal stools, constipation, dyspepsia/heartburn, taste disorder, myalgia, dyspnea, upper respiratory tract infection, decreased vitamin B₁₂ levels (7%), increased diaphoresis, flu-like syndrome, nail disorder

<1% (Limited to important or life-threatening): lactic acidosis, leukocytoclastic vasculitis, megaloblastic anemia, pneumonitis

Contraindications to the use of metformin are: hypersensitivity to metformin or any component of the formulation; renal disease or renal dysfunction (serum creatinine ≥ 1.5 mg/dL in males or ≥ 1.4 mg/dL in females) or abnormal creatinine clearance from any cause, including shock, acute myocardial infarction, or septicemia; acute or chronic metabolic acidosis with or without coma (including diabetic ketoacidosis)

Boxed warning: A boxed warning has been issued for metformin related to the risk of developing lactic acidosis. The risk is increased in patients with acute congestive heart failure, dehydration, excessive alcohol intake, hepatic or renal impairment, or sepsis. Conditions that increase the risk of lactic acidosis on metformin treatment are all exclusion criteria for this study. We plan to monitor closely for lactic acidosis and will remove a patient for symptomatic lactic acidosis or a rise in serum lactate to greater than two times the upper limit of normal or two times their baseline value to a value outside the normal range. If symptomatic lactic acidosis occurs in one patient the study will be stopped altogether if the independent safety officer determines that the occurrence is related to the study drug.

Iodinated contrast: Metformin therapy will be discontinued prior to or at the time of intravascular administration of iodinated contrast media (potential for acute alteration in renal function) should a subject require contrast during the course of this study. Metformin will be withheld for 48 hours after the radiologic study and restarted only after renal function has been confirmed as normal. Patients will be encouraged to delay any elective radiologic studies requiring iodinated contrast until the conclusion of the study.

Adherence: We will have twice weekly phone contact with participants for the first week and weekly thereafter; second we have study visits planned for face-to-face discussion of adherence every two weeks; third, as metformin is known to decrease HOMA-IR, this will be assessed at each time point for blood collection in the study; fifth, if side effects limit adherence, we will decrease dose to a

tolerable dose as described above. Finally, pill counts from bottles will be conducted at each visit.

Treatment of persistent hyperglycemia: Some patients enrolled in this study may have persistent hyperglycemia despite appropriate therapy with metformin. We have chosen this dosing regimen because it closely mimics what is used in clinical practice and thus is likely to have a high degree of success in treating not just insulin resistance but also hyperglycemia. In the unlikely event that this is not successful in maintaining euglycemia, we will consult with our collaborator in the Endocrine Division, Dr. Kevin Niswender. If further evaluation or treatment is warranted, we will refer for additional therapy as clinically indicated. Data analysis will be appropriately adjusted if patients require additional anti-diabetic therapy in conjunction with our statisticians.

Criteria for Study Withdrawal or Study Discontinuation: We plan to monitor closely for lactic acidosis and will remove a patient for symptomatic lactic acidosis. Additional criteria for patient removal will be: rise in serum lactate to greater than two times the upper limit of normal or doubling of the baseline value to a value that falls above the normal range. If the independent safety officer determines that symptomatic lactic acidosis occurs and is attributable to study drug in one patient the study will be stopped altogether. Patients with symptomatic hypoglycemia unresponsive to dose reduction may withdraw. Inability to tolerate metformin or hypersensitivity to drug will trigger withdrawal. Development of renal dysfunction that would preclude enrollment will require withdrawal. Patients who require additional anti-diabetic drugs prior to study completion will continue to be enrolled. Furthermore, the ISO has the ability to stop the study for safety concerns. Patients will not be withdrawn due to progression of their underlying disease as long as all other safety measures are met. Any patient who voluntarily withdraws or withdraws because of an aforementioned adverse event will be requested to allow us to continue to accrue clinical data for analysis, if possible.

Debriefing: At the time of each research visit, participants are debriefed and all questions about the study answered. This will include a review of the study aims, protocol, and any additional issues related to the study. Participants are also provided contact information if they have questions at any time.

B. Procedures for Minimizing Confidentiality Risks:

Risks related to patient confidentiality associated with storage of clinical data and samples, consent forms, questionnaire data, and study data are stored in secured files, either in locked file cabinets or in a locked room separate from medical records and coded such that all patient identifiers have been removed. Subjects are given a code that is electronically generated, and this code is used to identify the subjects in the database. Only approved study staff have access to the code that can identify the subjects. As an additional precaution all HIPAA

regulated information is stored in an electronic file separate from other study data (REDCap). Only approved study staff are given authorization to access the database. The Principal Investigator and the study coordinator determine approved study staff. Bio-specimens are processed and labeled with barcode labels that include the subjects electronically generated study code and date of sample collection. The bio-specimens are stored in locked freezers in the study Laboratory; only approved study staff has access to the keys for each freezer. Access to the electronic freezer inventory of the specimens is kept on a secure password protected computer in the study Laboratory. This inventory includes only the subjects' study code and is not linked to the subjects' identity within the freezer inventory program. Language on the written informed consent document will comply with standardized institutional language regarding human genetic information studies.

C. Reporting Adverse Events and Data Monitoring

As stated above, there is a low risk of adverse events with venipuncture, echocardiography, PET scan and IV placement. The risks of metformin are described above. If clinically important and unexpected adverse experiences occur, they will be recorded on the adverse event case report form. Investigators will report all serious, unexpected, and study-related adverse events to the ISO and the local Institutional Review Board in a timely manner.

The Vanderbilt IRB requires that the following events be reported to the IRB:

- a) Any event that requires prompt reporting to the sponsor, in accordance with the protocol (e.g., serious adverse events);
- b) Accidental or unintentional change to the IRB-approved protocol that involves risks or has the potential to recur;
- c) Deviation from the protocol taken without prior IRB review to eliminate apparent immediate hazard to a research participant;
- d) Publication in the literature, safety monitoring report including a Data and Safety Monitoring Report, interim result, or other finding that indicates an unexpected change to the risk/benefit ratio of the research;
- e) Adverse event that is both a serious adverse event and an unexpected adverse event, which in the Investigator's opinion is more likely than not to be related to the research procedures

Adverse event reporting: It is proposed that the investigators will grade events possibly related to the study in the following manner:

Adverse Event (AE) Grading Scale:

0= No Adverse Event or within normal limits

1= Mild Severity: Transient laboratory test alterations; discomforts noted but no disruption of daily activities; no therapy, or only symptomatic therapy required

2= Moderate Severity: Laboratory test alterations indicating injury without long-term risk; discomfort sufficient to modify normal daily activity; specific therapy required (i.e., more than symptomatic)

3= Serious Severity: Laboratory test indicating a serious health threat or permanent injury; incapacity, inability to work, inability to perform normal daily activity; hospitalization required or prolonged; emergency treatment required; life-threatening events; death

Adverse events of a serious severity thought to be related to the research protocol will be reported to the Vanderbilt IRB, the ISO and Drs. Hemnes and Brittain as soon as possible, but within 72 hours of occurrence and the specific study will cease to perform the suspect procedure until review.

- f) Breach in confidentiality that may involve risk to that individual or others;
- g) Complaint of a participant that indicates an unanticipated risk or which cannot be resolved by the research staff; or
- h) Other event that is unanticipated, involved risk to participants or others and was possibly related to the research procedures.

Data monitoring will routinely be done by Drs. Hemnes and Brittain and in response to the reports of serious adverse events.

Data Safety Monitoring Plan: An Independent Safety Officer (ISO) will monitor the trial. The function of this person, Dr. Todd Rice, is to provide objective review of study procedures as they relate to human subject safety and data quality. Dr. Rice will receive thorough reports of the progress of the study after the each patient is enrolled and generates study data. These reports will provide timely information regarding safety reporting, data quality and patient recruitment.

Dr. Rice will have the authority to modify the study protocol or terminate the study if he deems such actions to be warranted. The ISO will prepare summary reports to be provided to the Institutional Review Board and the investigators. A single episode of lactic acidosis without any other cause than metformin may be adequate to terminate the trial.

Drs. Hemnes or Brittain will also be asked to be available for rapid access by the investigators in the case of the need to evaluate serious adverse events or any other major unanticipated or safety related issues. If and when such events occur, Drs. Hemnes and Brittain will be provided with all of the available clinical data surrounding the clinical occurrence. During the conduct of the trial, all reports of serious adverse events will be provided to the ISO in a timely manner for review. During its regularly scheduled meetings, the ISO will also be provided with a list of non-serious adverse events organized by treatment group. The ISO will be charged with the prompt review of this information as well as with providing feedback as necessary.

Ethical Conduct in Human Research: Drs. Hemnes, Brittain, and other study personnel have completed the Web-based Collaborative RB training Initiative (CITI) course, passed the online CITI examination and have completed required subsequent refresher courses.

D. Potential Benefits to Study Participation:

No direct benefit to the study participant is expected. The potential benefits to science and mankind that may result from this study include the knowledge that we will obtain regarding the potential relationship between insulin resistance and pulmonary vascular disease and right heart function. The study may lead to more successful treatment of PAH. In the short term, patients with identified insulin resistance will be potentially protected from some of the immediate sequelae of insulin resistance and hyperglycemia.

Table 1. Schedule of Measures During Study Protocol

| Variable | Week 0 | Week 2 | Week 4 | Week 8 |
|----------------------------------|---------------|---------------|---------------|---------------|
| Education | X | | | |
| Medical History/Allergies | X | X | X | X |
| Height, weight, BP, pulse | X | X | X | X |
| Blood/Urine collection | X | X | X | X |
| 6 minute walk distance | X | X | X | X |
| PET (FDG, C11 acetate) | X | | | X |
| MRS (TG%, RVEF) | X | | | X |
| Echocardiography | X | | | X |

Figure 1. Study Schedule

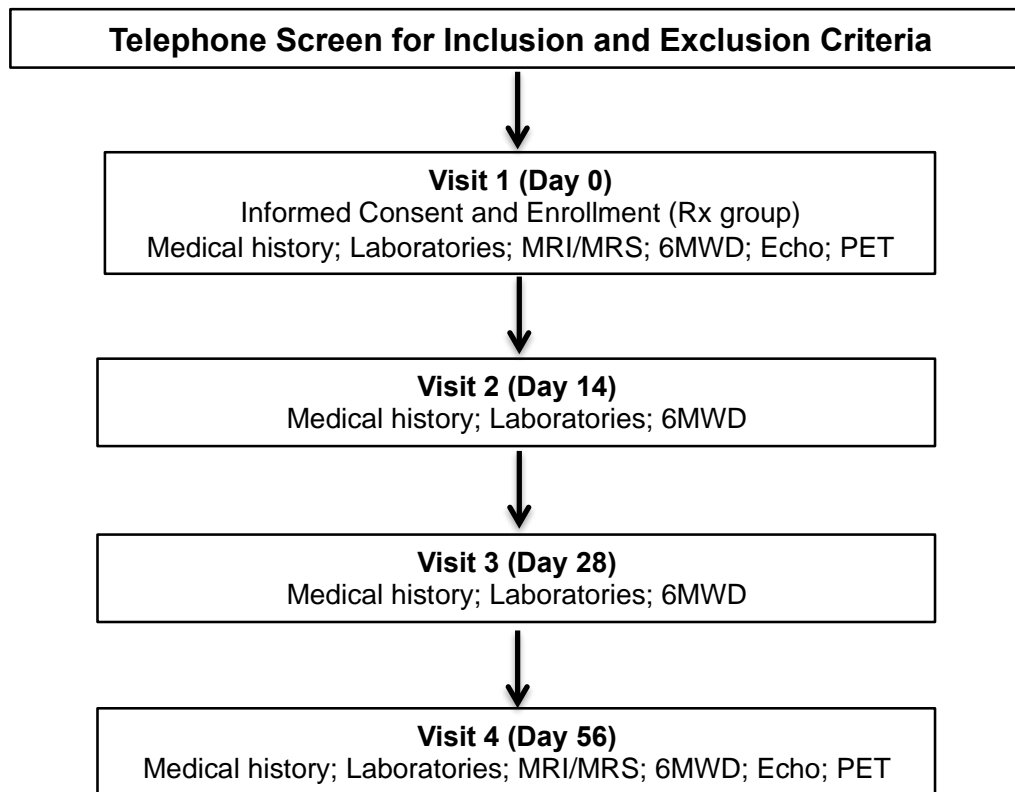


Table S1. Schedule of Measures During Study Protocol.

| Variable | Week 0 | Week 2 | Week 4 | Week 8 |
|----------------------------------|---------------|---------------|---------------|---------------|
| Education | X | | | |
| Medical History/Allergies | X | X | X | X |
| Height, weight, BP, pulse | X | X | X | X |
| Blood/Urine collection | X | X | X | X |
| 6 minute walk distance | X | X | X | X |
| PET (FDG, C11 acetate) | X | | | X |
| MRS (TG%, RVEF) | X | | | X |
| Echocardiography | X | | | X |

Table S2. Plasma Glucose, Insulin and Lipid Profiles Before and After Metformin.

| | Pre- | Post- | p value |
|---------------------------|-------------|-------------|---------|
| Total cholesterol (mg/dl) | 149.5 (29) | 151 (35) | 0.71 |
| HDL (mg/dl) | 50 (10) | 50 (11) | 0.93 |
| Triglycerides (mg/dl) | 103 (56) | 109 (52) | 0.49 |
| LDL-C (mg/dl) | 77 (25) | 80 (32) | 0.55 |
| TG/HDL ratio | 2.62 (1.69) | 2.28 (1.10) | 0.37 |
| Fasting glucose | 88 (11) | 86 (11) | 0.46 |
| Fasting insulin | 8.4 (5.8) | 7.7 (4.0) | 0.64 |

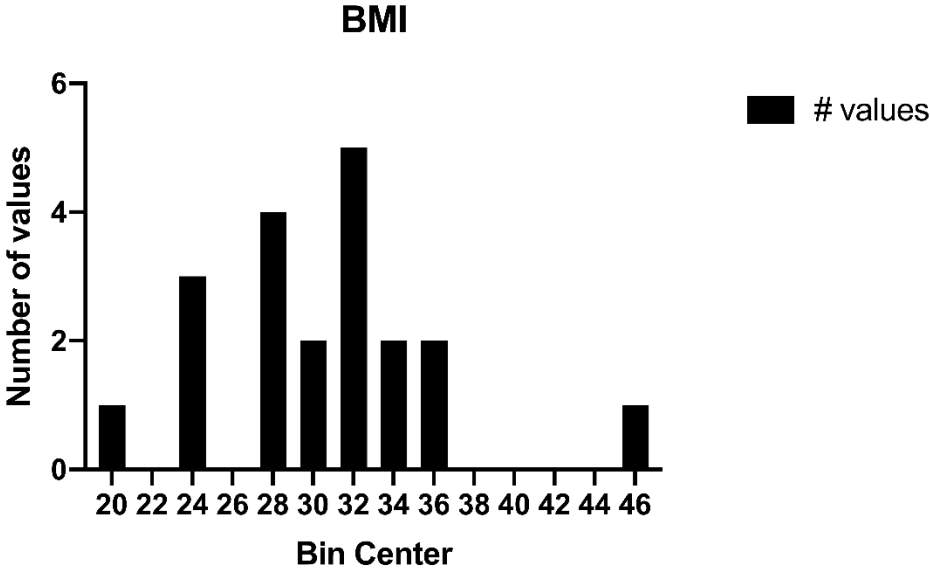
Data are presented as mean (SD)

Table S3. Metabolites significantly changed by metformin in patients with magnetic resonance spectroscopy data.

| Biochemical | Responders (% change from baseline) | Non-responders (% change from baseline) | p-value |
|--------------------------|--|--|----------------|
| 3,4-dihydroxybutyrate | -34.2 ± 7.45 | -8.3 ± 14.4 | 0.0159 |
| beta-sitosterol | 153.8 ± 77.37 | 34.1 ± 67.8 | 0.0195 |
| 2-piperidinone | -21.2 ± 49.1 | 132.9 ± 157.5 | 0.0159 |
| N-acetylputrescine | -8.2 ± 4.5 | -1.8 ± 5.3 | 0.0317 |
| 2-hydroxystearate | 57.1 ± 36.4 | 25.0 ± 80.7 | 0.0159 |
| Glucuronate | -38.1 ± 32.6 | 6.1 ± 18.8 | 0.0159 |
| heptenedioate (C7:1-DC)* | -9.9 ± 27.5 | 56.8 ± 85.5 | 0.0317 |

Data are presented as mean ± SD

Figure S1. Histogram of baseline BMI of study participants.



n=20

Figure S2. Effect of metformin on plasma oxidant stress markers.

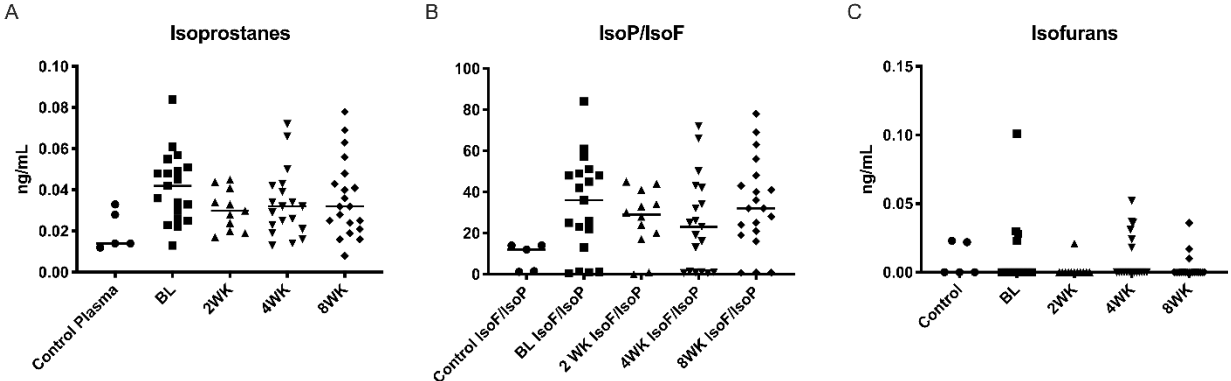


Figure S3. Fractional area change before and after metformin.

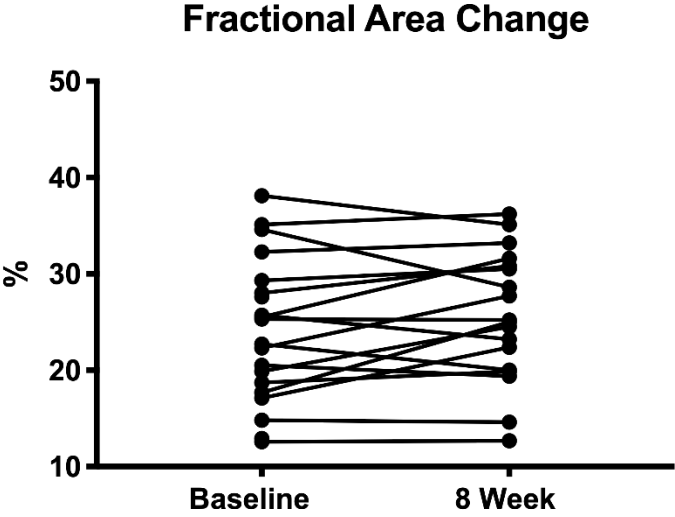
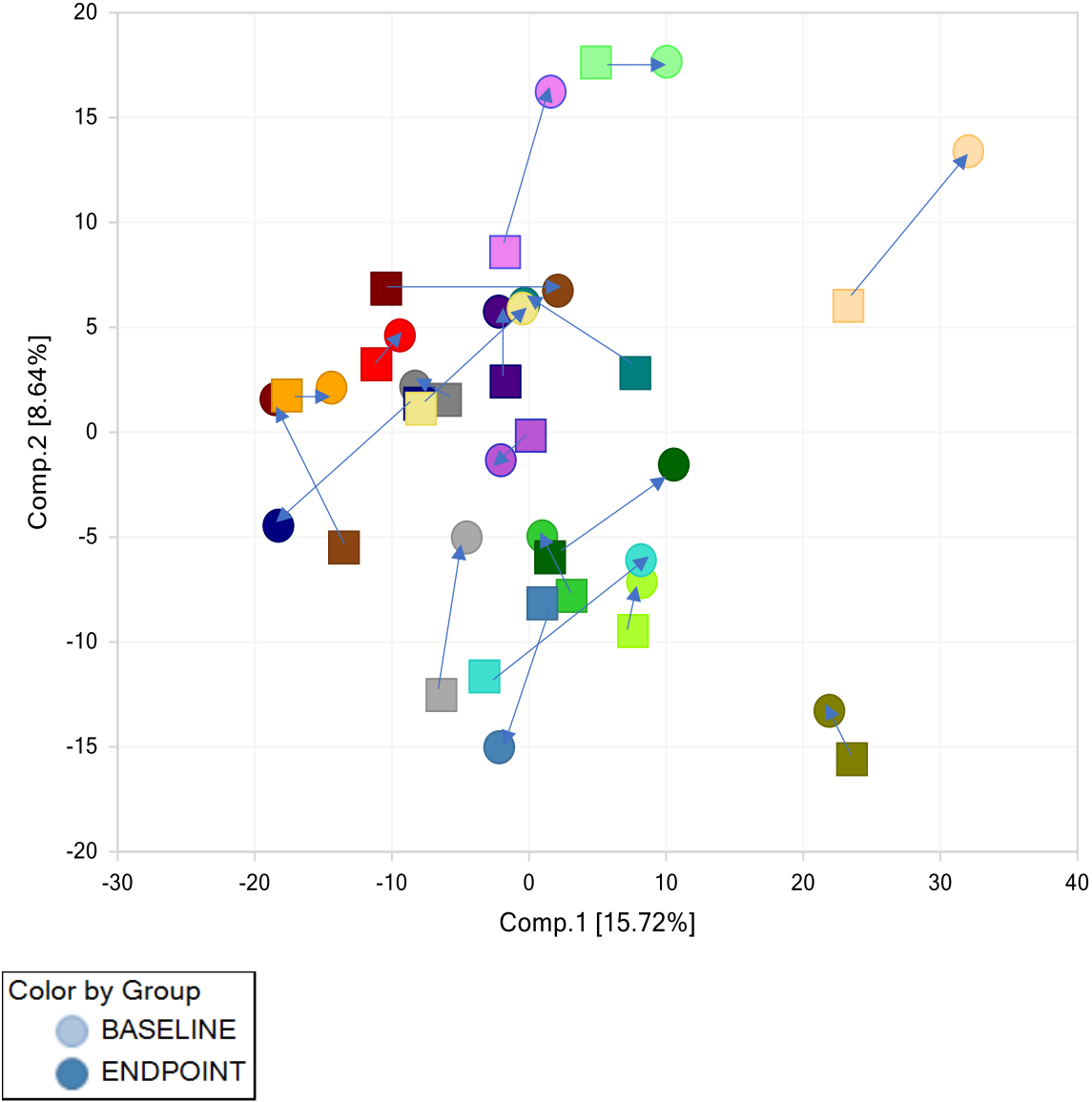


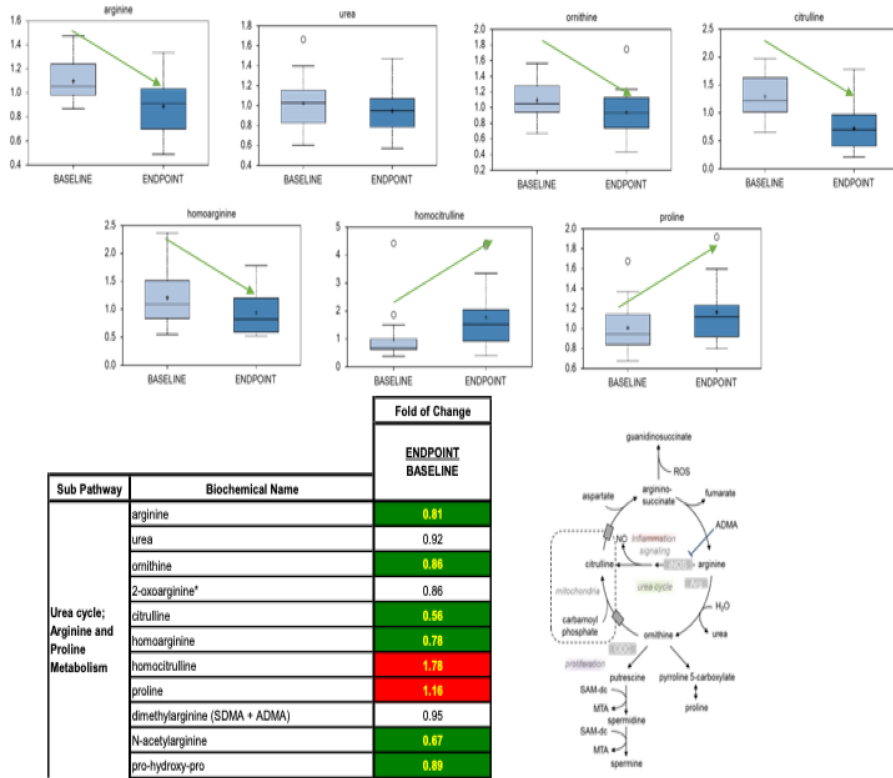
Figure S4. Change in plasma metabolome in individual patients before and after metformin exposure.



Comparison of baseline and end of study plasma metabolomic analysis in individual enrollees using principle components analysis. N=20

Figure S5.

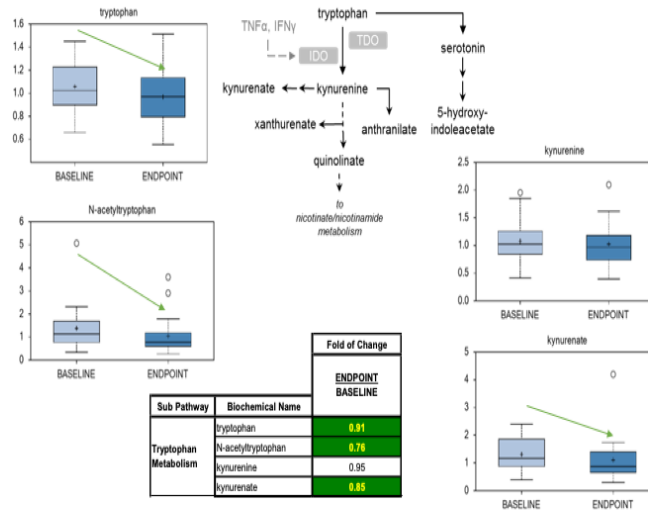
Metformin altered urea cycle metabolites in patients with PAH



In plasma from subjects before and after metformin therapy, there were significant alterations in plasma markers of the urea cycle. n=20

Figure S6.

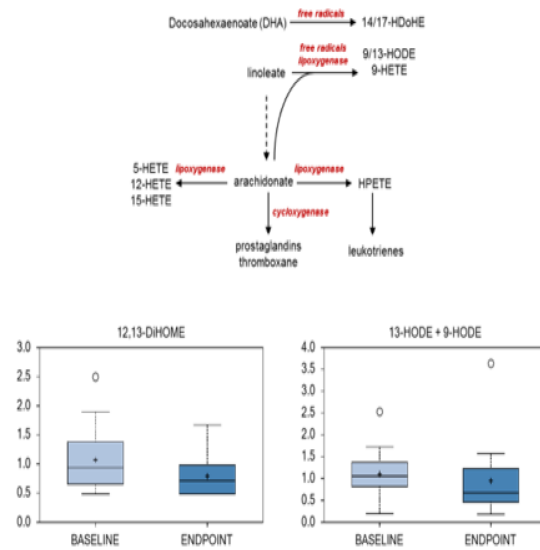
Metformin decreased markers of inflammation-tryptophan metabolism



In plasma from subjects before and after metformin therapy, there were significant alterations in plasma markers of inflammation and tryptophan metabolism. n=20

Figure S7.

Metformin decreased markers of inflammation



In plasma from subjects before and after metformin therapy, there were significant alterations in plasma markers of inflammation in the DiHOME pathway. n=20