

Metabolomic Insights in Pulmonary Arterial Hypertension

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Pulmonary hypertension (PH) has long been regarded as a rare disorder. This is true of pulmonary arterial hypertension (PAH), but raised pulmonary artery pressure can accompany many systemic diseases and there is increasing appreciation of an overlap in pathophysiology.¹ Indeed, recent detailed histology of the lungs from patients with PH associated with left heart failure show global pulmonary vascular remodeling involving arterioles as well as veins.² These observations challenge the current clinical classification of PH and inspire the application of “-omic” technologies to provide in-depth molecular phenotyping of patients and better understand the drivers of PH. One of these technologies is metabolomics, which has developed as a powerful means of identifying changes in metabolism in a wide range of diseases.³

The metabolome represents the entire composition of small molecular weight (<1500 Da) molecules in a biological sample such as tissue, plasma, or urine. These molecules include both endogenous compounds (eg, amino acids, short peptides, nucleic acids, fatty acids, lipids, amines, carbohydrates) and exogenous chemicals that are not naturally produced or expected to be found in the body (eg, drugs, pollutants, food additives, xenobiotics). Databases such as the Human Metabolome Database (www.hmdb.ca) currently contain over 110,000 metabolites; however, the number that have been identified and measured is

only a fraction of the compounds that are predicted to be present.⁴ No single “-omics” technology can encompass all the molecular events that lead to a disease such as PAH, but metabolites represent the “business end” of the genome, being closer to the phenotype than gene or protein expression, and metabolomics analysis offers a means of integrating variation at the genomic, epigenetic, transcriptomic, and proteomic levels. The metabolome also reflects environmental influences, being responsive to factors such as physical activity, diet, the microbiome and environmental exposures, and arguably the phenotype, the overall molecular expression of the state of health of an individual. So, a snapshot of an individual's metabolome provides a richer vocabulary to describe a person's state of health and inform disease status and its molecular drivers, including coexisting factors.

Measuring all metabolites in a single specimen has a number of potential challenges, including those due to differences in physical properties, concentration range, and stability. There are 2 main approaches, using either nontargeted or targeted metabolomics to detect as many compounds as possible or measure a selective group of metabolites. The methods most often employed rely on nuclear magnetic resonance (NMR) or mass spectrometry (MS) coupled with chromatography methods, such as gas or ultra-performance liquid chromatography

(GC or UPLC), and each technique has inherent advantages and disadvantages. NMR is less expensive, more stable, and does not destroy the sample, whereas MS techniques often detect higher numbers of metabolites with high sensitivity.³ These approaches are research based, but translational application at the bedside is not far away. Takats et al have developed an electrosurgical knife that tests evaporated tissues for metabolic cancer markers to aid surgeons in determining whether they have removed all of a tumor during an operation.⁵ The measurement of disease-related volatile organic metabolites in exhaled breath offers another opportunity for metabolomic profiling, with a view to earlier diagnosis and intervention.⁶

TISSUE METABOLOMICS AND TARGETED PLASMA METABOLITE STUDIES IN PAH

One of the first applications of metabolomics to samples from patients with PAH was performed on lung tissue.⁷ A comparison of 8 PAH lung samples collected at transplantation with 8 lobectomy samples identified increased glycolysis metabolites and altered fatty acid profiles, alongside changes in gene expression; the 4 genes that encode the enzymes fatty acetyl-CoA L1 (*ACSL1*), Acyl CoA dehydrogenases (*ACADM*), Acetyl-CoA acetyltransferase1 (*ACAT1*), and Acetyl-CoA carboxylase (*ACACA*), respectively, were all increased in PAH tissue. Tricarboxylic acid (TCA) cycle metabolites including citrate, succinate, cis-aconitate, and genes including *SUCLA2*, encoding succinate-CoA ligase, the gene encoding fumarate hydratase (*FH*), and the genes encoding aconitase (*IREB1*) and the second isoform of aconitase, iron responsive element

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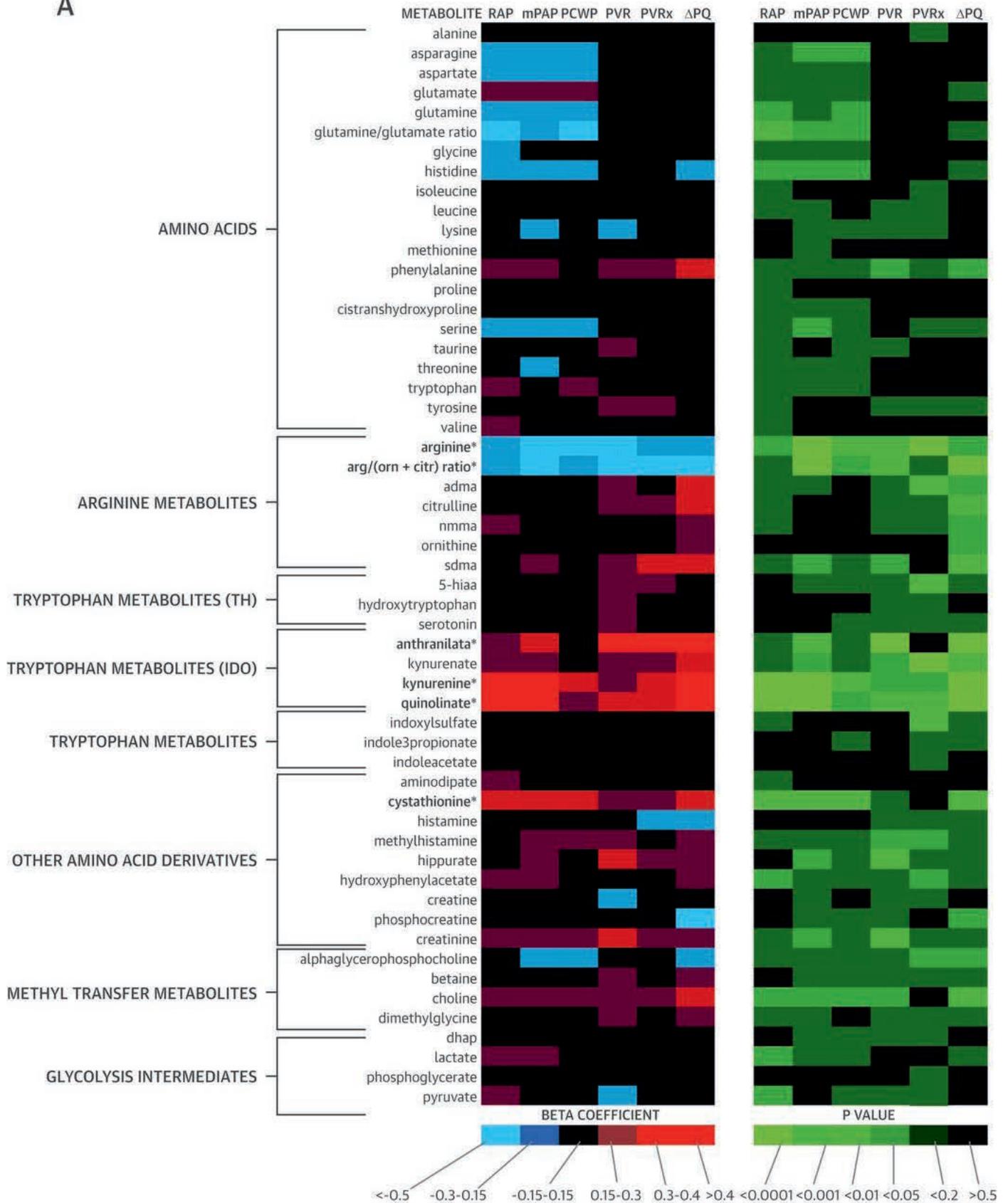


Figure 1: Reprinted from Lewis GD, Ngo D, Hemnes AR, et al. Metabolic profiling of right ventricular-pulmonary vascular function reveals circulating biomarkers of pulmonary hypertension. *J Am Coll Cardiol.* 2016;67(2):174-189, with permission from Elsevier.

binding protein 2 (*IREB2*), were all up-regulated in PAH tissues. Whether this reflects increased TCA cycle activity or a buildup of intermediates due to a block in the cycle cannot be concluded from these data, but clearly the energetics of pulmonary cells are disturbed in PAH patients. Previous data on alterations in metabolism driven by *BMPR2* mutations (the principle cause of heritable PAH⁸) in human pulmonary artery endothelial cells also demonstrated the relevance of energetics, but with some differences; several TCA intermediates were reduced, except citrate, suggesting a possible block downstream of citrate, and acylcarnitines and glutamate were also lower in cells expressing the mutant *BMPR2*.⁹ Both studies showed elevated gene expression of isocitrate dehydrogenase1 (*IDH1*) and Fessel et al suggested serum activity may be a biomarker of disease severity.⁹

A later analysis of the lung metabolite data demonstrated further abnormalities including increased sphingolipids, heme breakdown products (biliverdin, bilirubin), and arginine metabolism (leading to polyamine, putrescine, spermidine, and spermine production) in PAH.¹⁰ This was again paired with altered gene expression, including elevated arginase1 (*ARG1*), sphingomyelin synthase 2 (*SGMS2*), and N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2 (*ASAH2*) in PAH tissue.

A targeted study of dyspneic individuals where plasma concentrations of 105 metabolites were analyzed and related to hemodynamic measures, including resting right atrial pressure, mean pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), and PVR and PAP-flow response (DPQ) during exercise, identified indoleamine 2,3-dioxygenase (IDO)-dependent tryptophan metabolites (TMs; anthranilate, quinolinate, and kynurenine) (Figure 1) release higher levels in radial artery than proximal pulmonary artery in patients with PVR >2 Woods units and higher circulating levels in PAH patients than controls in an independent validation cohort.¹¹ Arginine and TCA cycle intermediates (aconitate, isocitrate, malate, and succinate methylmalonate) were associated with the severity of PH in the discovery analysis (n=71) and derived metabolite

scores were associated with mean PAP in a validation cohort (n=71).

The specificity of the IDO-TM changes was perhaps surprising, as the other pathway of tryptophan metabolism produces serotonin (via tryptophan hydroxylase). Perturbation of serotonin activity is linked with PAH^{12,13} but metabolites of this pathway were not associated with the measures studied.

The adult heart normally obtains 50% to 70% of its energy from fatty acid β -oxidation.¹⁴ In heart failure, circulating levels of fatty acids are elevated, but uptake to cardiac tissue can be inhibited by concomitantly elevated ketone bodies (β -hydroxybutyrate and acetoacetate). A study of nonesterified free fatty acids (FFAs) and acylcarnitines, which shuttle fatty acids across membranes, demonstrated FFAs were around twice as high in 19 patients with PAH than in 22 controls and long chain (defined as ≥ 14 carbons) acylcarnitines were also elevated.¹⁵ Higher levels of the long chain acylcarnitines, namely, palmitoylcarnitine, stearoylcarnitine, oleoylcarnitine, and linoleoylcarnitine, were associated with worse functional class. Proton magnetic resonance spectroscopy was used to demonstrate elevated lipid levels in right ventricular (RV) tissue from living patients with PAH (Figure 2). However, measurements of PAH RV tissue showed significant reductions in the levels of acylcarnitines compared to unmatched donor hearts and explanted hearts from patients with dilated cardiomyopathy (DCM), as diseased comparators. RV tissue from *BMPR2* mutant mice was significantly less able to metabolize long chain fatty acids than control tissue, suggesting that PAH RV tissue may have elevated fatty acids due to reduced processing to acylcarnitines. Indeed, elevated fatty acid levels were measured in PAH tissue, along with elevated ceramide, a potentially toxic alternative breakdown product.

UNTARGETED PLASMA METABOLOMICS IN PAH

Rhodes et al performed the first unbiased (in terms of target metabolite selection) screen of plasma metabolomics in patients with PAH using UPLC-MS methodologies provided by Metabolon Inc. (Durham, NC, USA).¹⁶

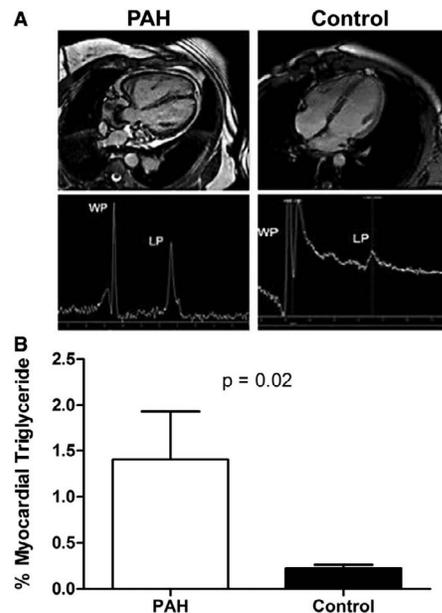


Figure 2: Reprinted from Brittain EL, Talati M, Fessel JP, et al. Fatty acid metabolic defects and right ventricular lipotoxicity in human pulmonary arterial hypertension. *Circulation*. 2016;133(20):1936-1944 [https://doi.org/10.1161/CIRCULATIONAHA.115.019351], with permission. Human proton magnetic resonance spectroscopy in PAH and controls. Nonfasting in vivo myocardial triglyceride content was quantified by using proton magnetic resonance spectroscopy in patients with PAH (n=6) in comparison with control subjects (n=8). Triglyceride content is expressed as a percent of triglyceride (%TG) in comparison with water in a 6-cm³ voxel placed in the interventricular septum. A, Representative cardiac MRI and proton magnetic resonance spectroscopy of a control subject demonstrates normal RV function and small LP. PAH patient has RV dilation and hypertrophy with a prominent LP. B, On average, %TG was 7-fold higher in PAH than in controls (1.4±1.3%TG vs 0.22±0.11%TG, P=.02). Interpatient reproducibility performed on 2 controls and 1 patient with PAH was within 0.05%TG. Analysis was performed by using the Mann-Whitney U test and data are presented as mean±SEM. Abbreviations: LP: lipid peak; PAH: pulmonary arterial hypertension; RV: right ventricle; SEM: standard error of the mean; WP: water peak.

Nonfasting plasma samples were provided by healthy controls (n=121 in 2 groups), disease controls (symptomatic patients referred to the PH clinical service at Hammersmith Hospital but found not to have PH, n=139 in 2 groups) and patients with idiopathic or heritable PAH (n=365 in a discovery and validation group from Hammersmith Hospital plus a further validation group from expert centers across

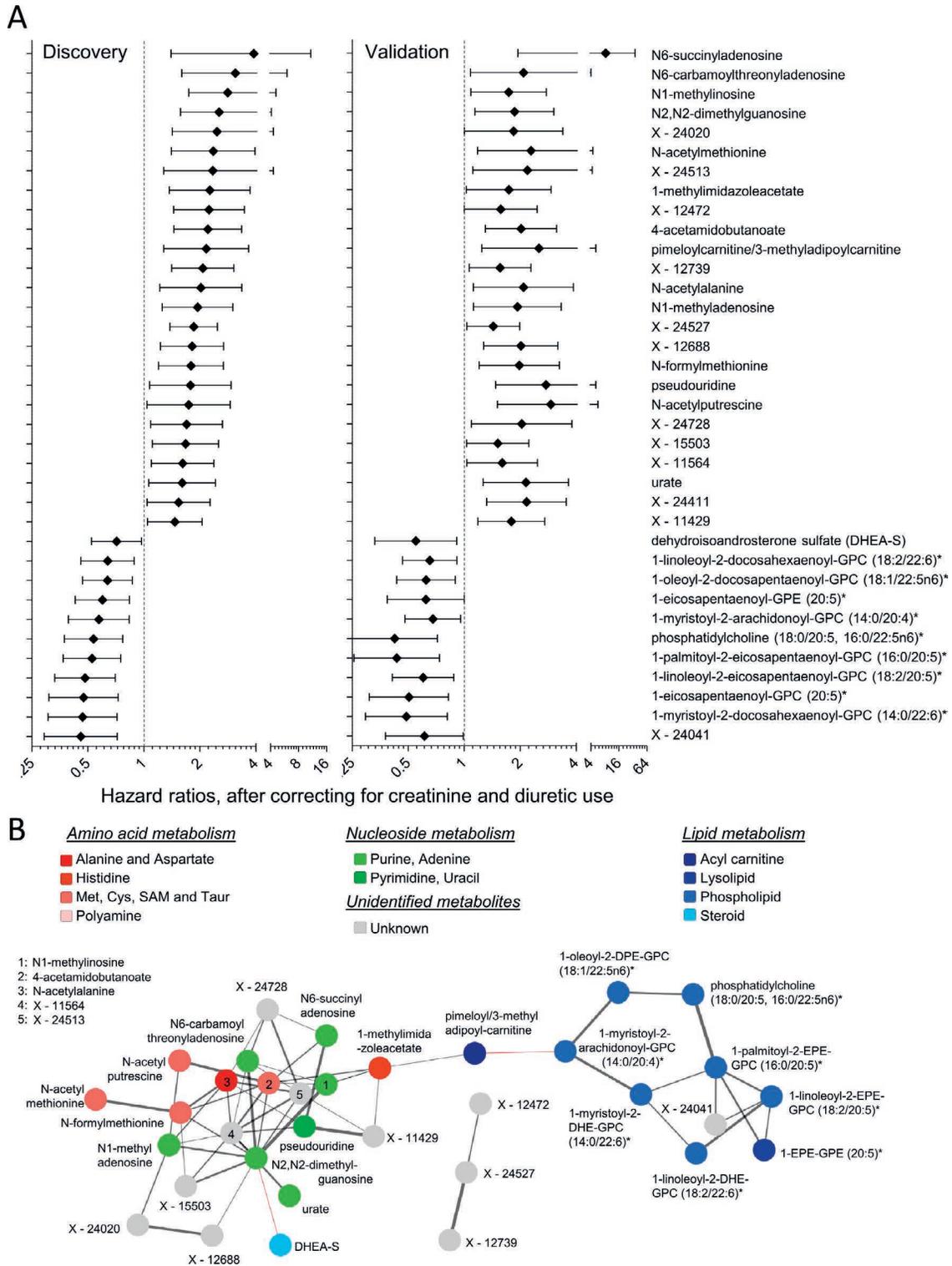


Figure 3: Reprinted from Rhodes CJ, Ghataorhe P, Wharton J, et al. Plasma metabolomics implicates modified transfer RNAs and altered bioenergetics in the outcomes of pulmonary arterial hypertension. *Circulation*. 2017;135:460-475 [https://doi.org/10.1161/CIRCULATIONAHA.116.024602], with permission. Prognostic metabolites independent of established risk factors. A, Hazard ratios after correcting for creatinine and diuretic use of 36 metabolites, which were prognostic in patients with PAH independent of RDW, NT-proBNP, and 6-minute walk distance. Hazard ratios indicate the risk of a change in each metabolite of 1 standard deviation, for ease of comparison. Patients of all ages were included in both discovery and validation survival analyses. B, Network analysis of the same 36 metabolites based on second order correlations. Line thickness indicates strength of correlations (all $P < .0001$). Red lines indicate negative correlations. *Probable metabolite identity, but unconfirmed (see methods). Abbreviations: DHE: docosahexaenoyl; DPE: docosapentaenoyl; DHEA-S: dehydroisoandrosterone sulfate; EPE: eicosapentaenoyl; GPC: glycerophosphocholine; GPE: glycerophosphoethanolamine.

the UK [The National Cohort Study of PAH, see ipahcohort.com]). Detected and measured in over 95% of samples were 686 biological metabolites. Plasma levels of 53 metabolites were significantly different in patients with PAH compared with healthy controls in all 3 cohorts after Bonferroni correction for multiple comparisons, and after further analyses correcting for potential confounders such as liver and renal function, age, sex, ethnicity, body mass index, and drug therapies. Twenty of these metabolites distinguished the PAH cohorts from the disease controls, and a network analysis identified the modified nucleoside, N₂,N₂-dimethylguanosine, and the TCA cycle intermediate, malate, as key hub metabolites. Discriminant scores based on just 7 and 4 of these metabolites were able to distinguish patients with PAH from healthy controls with 93% to 95% accuracy and from disease controls with 72% to 75% in validation cohorts. Interestingly, patients with PAH deemed to be vasoresponders, a subgroup who have relatively good clinical outcomes on calcium channel blocker therapy, had metabolite profiles more similar to healthy controls than patients with PAH. The patients in this study had established disease (ie, were not treatment naive), and it would be of great interest to study these metabolites before and after initiation of vasodilator therapy to observe whether vasoresponders have more “healthy” metabolomes at diagnosis or whether it is corrected by successful treatment.

Clustering analysis of these metabolites suggested that the most significant shifts in metabolite levels were associated with patients who died during follow-up. Consistent with this, 36 metabolites were prognostic in 2 cohorts of PAH patients, independent of NT-proBNP, 6-minute walk, and red cell distribution width data (Figure 3), a known highly prognostic combination.¹⁷ Serial samples from patients who died during follow-up were more likely to show a further increase in prognostic metabolites, including circulating modified nucleoside levels, than patients who survived, suggesting that monitoring metabolite levels over time could be useful in judging response to therapy.

The elevated circulating modified nucleosides (N₁-methylinosine and

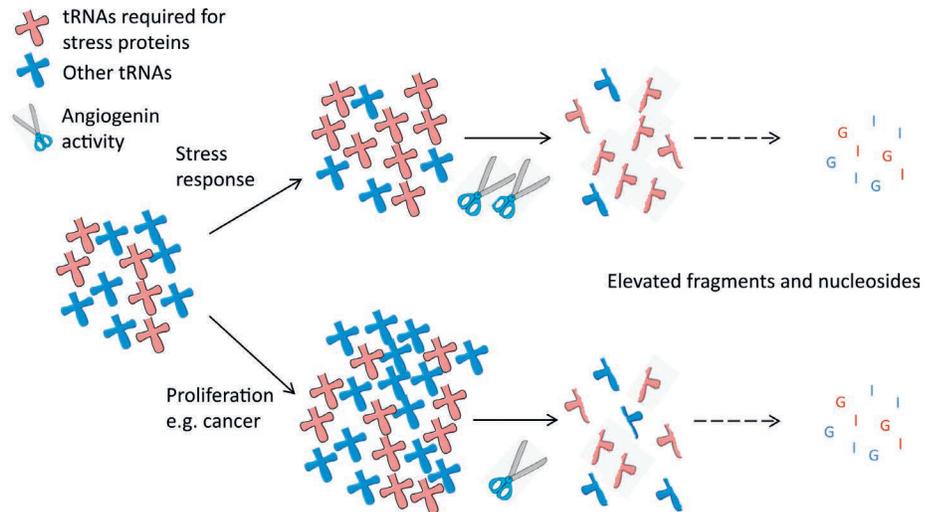
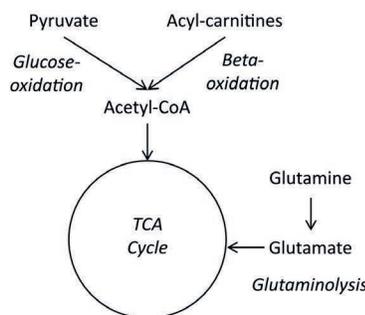


Figure 4: Causes of elevated circulating transfer RNA-derived modified nucleosides. In stress responses, such as renal failure, there is preferential expression of tRNAs required for the translation of stress proteins, and elevation of angiogenic. Angiogenic cleaves tRNAs, leading to the release of fragments into the plasma, which are further broken down to release modified nucleosides. In proliferative states, such as cancer, the cell replicative machinery is upregulated including expression of all tRNAs, such that normal levels of angiogenic and tRNA cleavage will consequently produce more fragments and hence higher plasma nucleoside levels.

Normal



IPAH

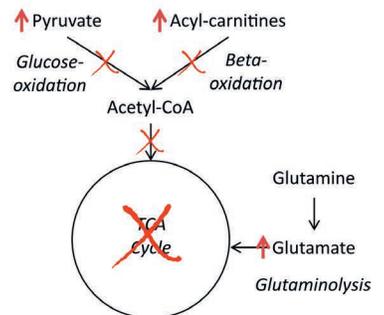


Figure 5: Altered energy metabolism in PAH schematic. In health, multiple pathways can feed into the tricarboxylic acid (TCA) cycle for production of the NADH/FADH₂ required for ATP production in the mitochondrial electron transport chain. These include glucose oxidation, beta-oxidation, and glutaminolysis. In PAH, buildup of the prerequisite molecules, pyruvate, acylcarnitines, and glutamate, is observed. This suggests an inability of the TCA cycle to match the pace of energy production required by proliferating vascular cells and cardiomyocytes facing an increased workload due to the elevated pulmonary artery pressures.

N₂,N₂-dimethylguanosine) originate from transfer RNA molecules and their release into the circulation during stress follows cleavage by the ribonuclease angiogenic (Figure 4).¹⁸ Elevated plasma angiogenic levels correlated with higher levels of modified nucleosides. Increased angiogenic levels have been reported in breath condensates¹⁹ of patients with PAH, which implicates the lung as a potential source. Furthermore, elevated tRNA nucleosides are observed in hyperproliferative cancers, most likely reflecting the general upregulation of the translational apparatus.^{20,21} This suggest-

ed that levels of these metabolites may be reporting on stressed, proliferative pulmonary vascular cells in patients with PAH.

Consistent with previous studies, we found alterations in several energetic metabolism pathways, including elevated acylcarnitines, glutamate, and altered TCA cycle intermediates. The buildup of precursors is consistent with dysfunction in the TCA cycle and/or the electron transport chain downstream, preventing cells from matching the energy demands forced upon them by the disease pathology (Figure 5). Not all

of the metabolites of the TCA cycle are easily quantified, so identification of the specific steps that may be affected was not possible in this study. As demonstrated by Brittain et al,¹⁵ circulating metabolite levels do not necessarily represent levels in relevant tissues and further study of specific pathways in pulmonary vascular and myocardial cells could illuminate these alterations.

There were reductions in multiple lipid species, including sphingomyelins and phosphatidylcholines and steroids including dehydroisoandrosterone-sulfate (DHEA-S) and metabolites, in patients with PAH, especially in those with poor clinical outcomes. Some of these may relate to developing insulin resistance but may also act more directly as sources of cellular signaling molecules, including eicosanoids, prostacyclin being the most relevant to PAH. In contrast to previous reports, circulating tryptophan metabolites were increased, but this tracked with a marker of liver dysfunction (bilirubin levels). This emphasizes the importance of interpreting circulating metabolomic profiles in the context of systems biology, and investigating the relevance to diseased tissue measurements can provide more direct evidence of action at the site of pathology.

USE AS A PROGNOSTIC MARKER

The observation that metabolite profiles are most disturbed in PAH patients with a poor clinical outcome has 2 implications. First, measurements of circulating metabolites in the clinic could provide useful information in terms of stratifying patients by prognosis. Second, medications capable of reversing the metabolic changes observed may be more likely to succeed in treating the disease. But these claims need to be examined prospectively and compared with prognostic risk equations such as the REVEAL score,²² and alternative candidates such as proteomic measurements from patients with PAH.²³ Establishing the role of these metabolites in the pathology of PAH is also key to adoption of measurements in clinical practice.

FUTURE DIRECTIONS

There is considerable interest in whether metabolomic profiles differentiate subclasses of PH. In the first instance, this will require broadening cohort studies to collect samples from patients presenting with a tentative diagnosis of PH. Studies showing altered metabolite profiles including blood tyrosine, ornithine, thyroid stimulating hormone, and phenylalanine levels in persistent PH of newborns already highlight that metabolic disturbances may occur early in multiple presentations of PH.²⁴ The question of tissue origin of the circulating metabolites of interest in PAH is also a pressing concern. Sampling of patients at multiple anatomic locations as well as studies in specific tissues and cells will help answer this. Efforts toward these goals are underway in the United Kingdom and in the United States as part of the PVDomics study.²⁵

CONCLUSION

High-throughput technologies have broadened our understanding of the metabolic disturbances in PAH far beyond the earlier studies focusing on the Warburg effect of switching to glycolytic metabolism as observed in cancers. Multiple studies have made complementary findings that encourage belief in the robustness of the associations between PAH disease burden and shifts in the way metabolites are transported and used, for example, in the failing right ventricle. The clinical utility of these measurements will depend on the development of easy-to-use assay formats that can be deployed in the clinic or in the longer term, assimilation of full metabolic profiling into clinical laboratories. Preclinical studies will define the importance and function of these metabolites, and therapeutic strategies to correct those implicated directly in the pathogenesis of PAH need to be explored.

References

1. Hoepfer MM, Humbert M, Souza R, et al. A global view of pulmonary hypertension. *Lancet Respir Med*. 2016;4(4):306-322.
2. Fayyaz AU, Edwards WD, Maleszewski JJ, et al. Global pulmonary vascular remodeling in pulmonary hypertension associated with heart

- failure and preserved or reduced ejection fraction. *Circulation*. 2018;137(17):1796-1810.
3. McGarrath RW, Crown SB, Zhang GF, Shah SH, Newgard CB. Cardiovascular metabolomics. *Circ Res*. 2018;122(9):1238-1258.
4. Wishart DS, Feunang YD, Marcu A, et al. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res*. 2018;46(D1):D608-D617.
5. Balog J, Szaniszló T, Schaefer KC, et al. Identification of biological tissues by rapid evaporative ionization mass spectrometry. *Anal Chem*. 2010;82(17):7343-7350.
6. Cohen-Kaminsky S, Nakhleh M, Perros F, et al. A proof of concept for the detection and classification of pulmonary arterial hypertension through breath analysis with a sensor array. *Am J Respir Crit Care Med*. 2013;188(6):756-759.
7. Zhao Y, Peng J, Lu C, et al. Metabolomic heterogeneity of pulmonary arterial hypertension. *PLoS One*. 2014;9(2):e88727.
8. Graf S, Haimel M, Bleda M, et al. Identification of rare sequence variation underlying heritable pulmonary arterial hypertension. *Nat Commun*. 2018;9(1):1416.
9. Fessel JP, Hamid R, Wittmann BM, et al. Metabolomic analysis of bone morphogenetic protein receptor type 2 mutations in human pulmonary endothelium reveals widespread metabolic reprogramming. *Pulm Circ*. 2012;2(2):201-213.
10. Zhao YD, Chu L, Lin K, et al. A biochemical approach to understand the pathogenesis of advanced pulmonary arterial hypertension: metabolomic profiles of arginine, sphingosine-1-phosphate, and heme of human lung. *PLoS One*. 2015;10(8):e0134958.
11. Lewis GD, Ngo D, Hemnes AR, et al. Metabolic profiling of right ventricular-pulmonary vascular function reveals circulating biomarkers of pulmonary hypertension. *J Am Coll Cardiol*. 2016;67(2):174-189.
12. Humbert M, Morrell NW, Archer SL, et al. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol*. 2004;43(12 suppl S):13S-24S.
13. MacLean MMR. The serotonin hypothesis in pulmonary hypertension revisited: targets for novel therapies (2017 Grover Conference Series). *Pulm Circ*. 2018;8(2):2045894018759125.
14. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev*. 2010;90(1):207-258.
15. Brittain EL, Talati M, Fessel JP, et al. Fatty acid metabolic defects and right ventricular lipotoxicity in human pulmonary arterial hypertension. *Circulation*. 2016;133(20):1936-1944.
16. Rhodes CJ, Ghataorhe P, Wharton J, et al. Plasma metabolomics implicates modified transfer RNAs and altered bioenergetics in the outcomes of pulmonary arterial hypertension. *Circulation*. 2017;135(5):460-475.

17. Rhodes CJ, Wharton J, Howard LS, Gibbs JS, Wilkins MR. Red cell distribution width outperforms other potential circulating biomarkers in predicting survival in idiopathic pulmonary arterial hypertension. *Heart*. 2011;97(13):1054-1060.
18. Kirchner S, Ignatova Z. Emerging roles of tRNA in adaptive translation, signaling dynamics and disease. *Nat Rev Genet*. 2015;16(2):98-112.
19. Seyfarth HJ, Sack U, Gessner C, Wirtz H. Angiogenin, bFGF and VEGF: angiogenic markers in breath condensate of patients with pulmonary hypertension. *Pneumologie*. 2015;69(4):207-211.
20. Anderson P, Ivanov P. tRNA fragments in human health and disease. *FEBS Lett*. 2014;588(23):4297-4304.
21. Waalkes TP, Gehrke CW, Zumwalt RW, et al. The urinary excretion of nucleosides of ribonucleic acid by patients with advanced cancer. *Cancer*. 1975;36(2):390-398.
22. Benza RL, Miller DP, Gomberg-Maitland M, et al. Predicting survival in pulmonary arterial hypertension: insights from the Registry to Evaluate Early and Long-Term Pulmonary Arterial Hypertension Disease Management (REVEAL). *Circulation*. 2010;122(2):164-172.
23. Rhodes CJ, Wharton J, Ghataorhe P, et al. Plasma proteome analysis in patients with pulmonary arterial hypertension: an observational cohort study. *Lancet Respir Med*. 2017;5(9):717-726.
24. Steurer MA, Oltman S, Baer RJ, et al. Altered metabolites in newborns with persistent pulmonary hypertension. *Pediatr Res*. Published online: June 12, 2018. DOI: 10.1038/s41390-018-0023-y.
25. Hemnes AR, Beck GJ, Newman JH, et al. PVDOMICS: A multi-center study to improve understanding of pulmonary vascular disease through phenomics. *Circ Res*. 2017;121(10):1136-1139.