Trans-right ventricle and transpulmonary metabolite gradients in human pulmonary arterial hypertension

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ABSTRACT

Objective While metabolic dysfunction occurs in several pulmonary arterial hypertension (PAH) animal models, its role in the human hypertensive right ventricle (RV) and lung is not well characterised. We investigated whether circulating metabolite concentrations differ across the hypertensive RV and/or the pulmonary circulation, and correlate with invasive haemodynamic/echocardiographic variables in patients with PAH.

Methods Prospective EDTA blood collection during cardiac catheterisation from the superior vena cava (SVC), pulmonary artery (PA) and ascending aorta (AAO) in children with PAH (no shunt) and non-PAH controls (Con), followed by unbiased screens of 427 metabolites and 836 lipid species and fatty acids (FAs) in blood plasma (Metabolon and Lipidixy platforms). Metabolite concentrations were correlated with echocardiographic and invasive haemodynamic variables.

Results Metabolomics/lipidomics analysis of differential concentrations (false discovery rate<0.15) revealed several metabolite gradients in the trans-RV (PA vs SVC) setting. Notably, dicarboxylic acids (eg, octadecanoic acid: fold change (FC) Control=0.77, FC_PAH=1.09, p value=0.044) and acylcarnitines (eg, stearoylcarnitine: FC_Control=0.74, FC_PAH=1.21, p value=0.058) were observed. Differentially regulated metabolites were also found in the transpulmonary (AAO vs PA) setting and between-group comparisons, that is, in the SVC (PAH-SVC vs Con-SVC), PA and AAO. Importantly, the differential PAH metabolite concentrations correlated with numerous outcome-relevant variables (eg, tricuspid annular plane systolic excursion, pulmonary vascular resistance).

Conclusions In PAH, trans-RV and transpulmonary metabolite gradients exist and correlate with haemodynamic determinants of clinical outcome. The most pronounced differential trans-RV gradients are known to be involved in lipid metabolism/lipotoxicity, that is, accumulation of long chain FAs. The identified accumulation of dicarboxylic acids and acylcarnitines likely indicates impaired β-oxidation in the hypertensive RV and represents emerging biomarkers and therapeutic targets in PAH.

INTRODUCTION Pulmonary arterial hypertension (PAH) is a progressive, fatal disease characterised by obliteration of pulmonary arterioles leading to a vicious cycle of increased pulmonary arterial pressure (PAP), pulmonary vascular resistance (PVR) and right ventricular (RV) pressure overload. The sequelae are RV hypertrophy (RVH), dilation and dysfunction, left ventricular (LV) compression/underfilling, RV capillary rarefication, decreased coronary perfusion (ischaemia, fibrosis) and ultimately RV failure. The pathobiological processes in PAH include altered gene expression, epigenetic changes, metabolic dysfunction and inflammation in the heart and pulmonary vasculature, but also organs and tissues outside the chest.

To date, no universally effective therapies are available, and ~25%-50% of PAH patients die within 5 years after diagnosis.

Previous work by others attempted to address possible transpulmonary biomarker gradients of circulating cyclic guanosine monophosphate, peptides (endothelin-1 and B-type natriuretic peptide) and proteins (IL-6, Platelet-Derived Growth Factor BB, Transforming Growth Factor beta1, Vascular Endothelial Growth Factor) in PAH. However, despite its limitations, sole right heart catheterisation is frequently performed at PH centres, so that, most of the above studies used either peripheral arterial blood or even pulmonary arterial wedge specimen as ‘post lung samples’. Most of the very few previously identified molecules with ‘differential transpulmonary concentrations’ were not associated with prognostic haemodynamic variables in these studies.

Although RV size and function are the major determinants of clinical outcome in PAH, and heart failure with preserved ejection fraction, trans-RV biomarker gradients have not been studied at all in children or adults with PAH, or any other cardiovascular disease (CVD), so far.

A few studies identified venous metabolites as potential biomarkers for PAH or CVD-related mortality. Circulating metabolites, particularly fatty acids (FAs), have been shown to reflect cardiac metabolic defects, and deteriorating ventricular function in left heart failure in mice. However, to our knowledge, no unbiased screens of trans-RV or transpulmonary metabolite gradients have been pursued in human cardiovascular disease so far. We hypothesised there is differential release or uptake of certain metabolites across both the hypertensive RV and lung, in the circulation of PAH versus non-PAH patients. We further hypothesised that such metabolic alterations are most likely due to high RV pressure afterload, pulmonary arterial shear stress (high PVR), RV-PA uncoupling/decreased RV efficiency, or other pathophysiological changes characteristic for PAH, and thus correlate with...
Table 1  Characteristics of control subjects and PAH patients studied

| Demographics                                      | Control (non-PAH) (n=8) | PAH (n=8) | P value |
|---------------------------------------------------|-------------------------|-----------|---------|
| Age, years (mean, range)                          | 6.4 (0.4–17)            | 7.9 (0.6–18) | n.s. (0.5627) |
| Male sex, n (%)                                   | 4 (50)                  | 3 (38)    |         |
| Height (m)                                        | 113.5±16.0              | 118.9±15.6 | n.s. (0.7209) |
| Weight (kg)                                       | 24.1±6.3                | 29.1±8.2  | n.s. (0.9163) |
| BSA (m²)                                          | 0.86±0.18               | 0.95±0.20 | n.s. (0.8330) |
| Disease subtypes (n)                              |                         |           |         |
| Mild to moderate LVOTO (6), mediastinal teratoma (1), portal vein stenosis (1) |           |           |         |

**Key haemodynamics**

**Cardiac catheterisation**

|                   | Control (non-PAH) | PAH | P value |
|-------------------|-------------------|-----|---------|
| sPAP (mm Hg)      | 23.4±2.5          | 68.8±11.4 | 0.0024 |
| mPAP (mm Hg)      | 17.3±2.2          | 60.4±8.9  | 0.0009 |
| dPAP (mm Hg)      | 12.3±2.1          | 38.4±5.3  | 0.0052 |
| mPAPlmSAP         | 0.28±0.03         | 0.82±0.12 | 0.0003 |
| mTPG (mm Hg)      | 6.4±1.1           | 51.5±9.2  | 0.0014 |
| dTPG (mm Hg)      | 1.6±0.6           | 35.5±8.0  | 0.0033 |
| PVRI (WU·m⁻²)     | 1.69±0.30         | 16.3±3.6  | 0.0003 |
| PVRSVR            | 0.11±0.02         | 0.82±0.14 | 0.0012 |
| Qp                 | 4.08±0.36         | 3.64±0.52 | n.s. (0.5054) |
| Qs                 | 4.46±0.42         | 3.78±0.60 | n.s. (0.3823) |
| Qp/Qs              | 0.92±0.04         | 0.99±0.04 | n.s. (0.2823) |
| mRAP (mm Hg)      | 5.5±6.0           | 6.6±1.2   | n.s. (0.1605) |

**Echocardiography**

|                   | Control (non-PAH) | PAH | P value |
|-------------------|-------------------|-----|---------|
| RVAWD (cm)        | 0.34±0.03         | 0.72±0.09 | 0.0022 |
| RVEDD (cm)        | 1.26±0.21         | 2.27±0.38 | n.s. (0.1098) |
| TAPSE (cm)        | 1.95±0.09         | 1.62±0.12 | n.s. (0.1111) |
| LVEF (%)          | 74.7±2.2  | 67.7±3.6   | n.s. (0.1419) |

Values are presented as mean±SEM. A Mann-Whitney U test was applied. P<0.05 was considered significant. All PAH patients with repaired CHD (PAH-CHD) had the repair >12 months prior to cardiac catheterisation. Two of the PAH patients had trisomy 21 (all with PAH-repaired CHD; patient ID #7 and #8 in online supplementary table S1). BSA, body surface area; CHD, congenital heart disease; dPAP, diastolic pulmonary arterial pressure; dTPG, diastolic transpulmonary pressure gradient; IPAP, idiopathic PAH; LVEF, left ventricular ejection fraction; LVPOT, left ventricular outflow tract obstruction; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; mSAP, mean systemic arterial pressure; mVRI, mean transpulmonary gradient; mVRI, mean transpulmonary gradient; PAH, pulmonary arterial hypertension; PFO, patent foramen ovale; PH, pulmonary hypertension; PVPR, pulmonary vascular resistance; PVR, PVR index; Qp, pulmonary blood flow; Qs, systemic blood flow; Qs, systemic flow index; RVEDD, right ventricular end-diastolic diameter; RPAP, systolic pulmonary arterial pressure; SVR, systemic vascular resistance; TAPSE, tricuspid annular plane systolic excursion; WU, Wood units.

Figure 1  Catheter positions during cardiac catheterisation used in this study. Selected metabolites in the trans-RV and transpulmonary gradients and the direction of the change are shown. The changes in octadecanedioate suggest disruption of peroxisomal β-oxidation and a compensatory shift to γ-oxidation of fatty acids in the hypertensive RV of PAH patients. Accumulation of stearoylcarnitine in PAH RV indicates incomplete mitochondrial fatty acid oxidation. Accumulation of both octadecanedioate and stearoylcarnitine contribute to lipotoxicity in PAH. A more complete overview of the trans-RV metabolic changes and their likely effects in PAH is presented in figure 6. AA0, ascending aorta; Con, control; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine; PA, pulmonary artery (right or left); PAH, pulmonary arterial hypertension; PC, phosphatidylcholine; RV, right ventricle; SVC, superior vena cava; Trans-PC, transpulmonary circulation.

**METHODS**

**Study population and study design**

This was a prospective study enrolling consecutively eight PAH patients and eight non-PAH control subjects, all strictly meeting entry criteria, from August 2013 to July 2015. Paediatric PAH was defined as per 2015/2016 international guidelines.1,3 Non-PAH control patients had mild to moderate LV outflow tract obstruction (n=6), benign mediastinal teratoma (n=1), or portal vein stenosis (n=1), see table 1 and online supplementary table S1. We excluded subjects with any intracardiac or extracardiac shunt, end-stage PAH, and/or any recent clinical instability or infection. EDTA blood was collected nearly simultaneously at three sites (superior vena cava (SVC); pulmonary artery (PA); ascending aorta (AA0)). First, we used the Metabolon platform to profile 427 metabolites. Subsequently, we performed lipidomics analysis (using the Lipidyzer platform) in a cohort of eight PAH patients and nine controls (online supplementary tables S2 and S3), which largely overlapped with the subjects used for the Metabolon study. The analysis included 836 lipid species and their corresponding FAs; the latter are reported in the following format throughout the text: Lipid (FA carbon atoms: unsaturated double bonds). We maintained platform-specific abbreviations for (glycero-)phosphatidyl and (glycero-)phosphoethanolamine lipids as PC and PE, respectively, for lipids identified by Lipidyzer and GPC and GPE for lipids identified by Metabolon.

**Materials and methods**

A detailed description of the study design, sample collection, methods and statistical analysis can be found in the online supplement. See also figure 1.
Several metabolites have differential trans-RV (PA vs SVC) gradients in paediatric PAH (vs non-PAH control). Statistical test: Wald $\chi^2$ test (linear mixed effects models). Data filtration: Metabolites with a missing value in at least one of the three catheterisation sites were removed from the gradient analysis. Outliers were removed using a recursive removal procedure for the outliers identified using Grubb’s test as described in the statistics section of the online supplement. (A) Step-up (FC=2.31) in PAH and step-down (FC=0.70) in controls for 1-palmitoyl-GPE (16:0) levels (FDR-adjusted p value=0.0133); raw data (normalised ion counts) for 1-palmitoyl-GPE (16:0) levels in all three catheterisation sites; correlation of 1-palmitoyl-GPE (16:0) levels with haemodynamics (TAPSE). (B) Step-up (FC=1.48) in PAH and step-down (FC=0.78) in controls for 1-oleoyl-GPE (18:1) levels (FDR-adjusted p value=0.0767); raw data (normalised ion counts) for 1-oleoyl-GPE (18:1) levels in all three catheterisation sites; correlation of 1-oleoyl-GPE (18:1) levels with haemodynamics (TAPSE). (C) No change (FC=1.09) in PAH and step-down (FC=0.77) in controls for octadecanedioate levels (FDR-adjusted p value=0.0439); raw data (normalised ion counts) for octadecanedioate levels in all three catheterisation sites; correlation of 11-oleoyl-GPE (18:1) levels with haemodynamics (TAPSE). (D) Step-up (FC=1.21) in PAH and step-down (FC=0.74) in controls for stearoylcarnitine levels (FDR-adjusted p value=0.0582); raw data (normalised ion counts) for stearoylcarnitine levels in all three catheterisation sites; correlation of stearoylcarnitine levels with haemodynamics (sPAP). AAO, ascending aorta; Con, control; FC, fold change; FDR, false discovery rate; GPE, glycerophosphoethanolamine; PA, pulmonary artery; PAH, pulmonary arterial hypertension; RV, right ventricle; sPAP, systolic pulmonary arterial pressure; SVC, superior vena cava; TAPSE, tricuspid annular plane systolic excursion.
Several lipids have differential trans-RV (PA vs SVC) and transpulmonary (AAO vs PA) gradients in paediatric PAH (vs non-PAH control). Volcano plots (A and B) show pairs of lipids with statistically significant fold change differences. Interestingly, all such lipids had a step-up in PAH and a step-down in controls trans-RV (A), while the fold change direction was reversed (PAH down, non-PAH controls up) for the lipids with significantly different gradients transpulmonary (B). Statistical test: Wald $\chi^2$ test (linear mixed effects models). Data filtration: see legend for figure 1. AAO, ascending aorta; CE, cholesterol ester; DAG, diacylglycerol; FA, fatty acid; FFA, free FA; FDR, false discovery rate; FC, fold change; PAH, pulmonary arterial hypertension; PC, phosphatidylcholine; RV, right ventricle; SVC, superior vena cava; TAG, triacylglycerol; Trans-PC, transpulmonary circulation.

**Ethics statement**

All cardiac catheterisations were clinically indicated. Informed consent for study participation was obtained from the legal caregivers.

**Patient and public involvement**

It was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination of our research.

**RESULTS**

**Demographic and clinical characteristics**

Demographic and clinical characteristics of subjects for the Metabodyzer (lipidomics) study is shown in online supplementary table S2. Detailed information on each individual in the study including age, weight, body surface area (BSA), WHO functional class, and medication is provided in online supplementary table S1. PAH and non-PAH study groups were well-matched with respect to age, gender, height, weight and BSA (table 1).

**Phenotype discrimination in metabolite profiles of each experimental setting (principal component analysis)**

To assess the impact of metabolite variability on phenotype discrimination in each experimental setting (trans-RV and transpulmonary) and per individual catheterisation sites (SVC, PA, AAO) we performed principal component analysis as described in the online supplement. The results indicate adequate phenotype separation in each setting (online supplementary figures S1 and S3), which was better for the trans-RV than the transpulmonary fold changes.

**Differential trans-RV gradients (PAH vs control)**

are dominated by ‘lipid metabolites’ and correlate with invasive haemodynamic and echocardiographic variables

We identified 12 metabolites with significantly different trans-RV (PA vs SVC) plasma concentration gradients (PAH vs control; false discovery rate (FDR)<0.15). Four of these metabolites with the most pronounced differential gradients and probable biological relevance are shown in figures 1 and 2. These are glycerophosphoethanolamines (GPE) (1-palmitoyl-GPE (16:0) and 1-oleoyl-GPE (18:1)), a dicarboxylic acid (octadecanedioate) and an acylcarnitine (stearylcarcitnine), all of which are involved in lipid and phospholipid metabolism. All four of the above metabolites showed a step-up from SVC to PA in PAH patients, and/or the levels dropped from SVC to PA in controls only (step-down in controls; figure 1).

Comparison of the trans-RV log2 fold changes (for the four metabolites presented in figure 2) with key haemodynamic and echerographical variables (defined in table 1) revealed several important correlations, the strongest of which are shown in table 2 and online supplementary table S4, and include tricuspid annular plane systolic excursion (TAPSE), right ventricular anterior wall diameter (RVAWD), systolic pulmonary arterial pressure (sPAP), mean pulmonary arterial pressure (mPAP), PVR index (PVRi), etc. Overall, plasma levels of the aforementioned four ‘lipid metabolites’ (1-palmitoyl-GPE (16:0), 1-oleoyl-GPE (18:1), octadecanedioate, stearylcarcitnine), positively correlated with haemodynamic indicators of RV pressure afterload and PVD severity (sPAP, mPAP, PVR), and negatively with systolic longitudinal RV function (TAPSE).

The lipidomics analysis of the trans-RV gradients revealed that seven triacylglycerols (TAGs), one diacylglycerol (DAG(FA22:6)) and one cholesterol ester (CE20:2) had a step-up in PAH and a step-down in controls (figure 3A). The carbon chain lengths of their FA groups ranged between 14 and 20.

**Differential transpulmonary gradients (PAH vs control)**

of several circulating metabolites exist and correlate with invasive haemodynamic and echocardiographic variables

We identified seven metabolites with significantly (FDR<0.15) different levels (PAH vs control) across the pulmonary circulation (AAO vs PA). The transpulmonary plasma concentration gradients of four of these metabolites, that is, N-acetylcarnosine (step up in PAH), 2-palmitoyl-glycerophosphocholine (16:0) (step down in PAH), N-acetylcarnosine (step down in PAH) and azelate (nonanediolate) (step down in PAH) along with their correlation to haemodynamic and echocardiographic variables are shown in figure 4 (presented metabolites were selected based on function and effect size). Further discussion of metabolite
Several metabolites have differential transpulmonary (AAO vs PA) gradients in paediatric PAH (vs non-PAH control). Statistical test: Wald $\chi^2$ test (linear mixed effects models). Data filtration: see legend for figure 1. (A) Step-up (FC=1.53) in PAH and step-down (FC=0.71) in controls for N6-acetyllysine levels (FDR-adjusted $p$ value=0.0476); raw data (normalised ion counts) for N6-acetyllysine levels in all three catheterisation sites; correlation of N6-acetyllysine levels with haemodynamics (dTPG). (B) Step-down (FC=0.83) in PAH and step-up (FC=1.22) in controls for 2-palmitoyl-GPC (16:0) levels (FDR-adjusted $p$ value=0.0184); raw data (normalised ion counts) for 2-palmitoyl-GPC (16:0) levels in all three catheterisation sites; correlation of 2-palmitoyl-GPC (16:0) levels with haemodynamics (PVR/SVR). (C) Step-down (FC=0.72) in PAH and no change (FC=1.05) in controls for N-acetylcarnosine levels (FDR-adjusted $p$ value=0.0683); raw data (normalised ion counts) for N-acetylcarnosine levels in all three catheterisation sites; correlation of N-acetylcarnosine levels with haemodynamics (PAWP). (D) Step-down (FC=0.42) in PAH and step-up (FC=1.22) in controls for azelate (nonanedioate) levels (FDR-adjusted $p$ value=0.0006); raw data (normalised ion counts) for azelate (nonanedioate) levels in all three catheterisation sites; correlation of azelate (nonanedioate) levels with haemodynamics (dTPG). AAO, ascending aorta; Con, control; dTPG, diastolic transpulmonary pressure gradient; FC, fold change; FDR, false discovery rate; GPC, glycerophosphocholine; PA, pulmonary artery; PAH, pulmonary arterial hypertension; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance; SVC, superior vena cava.
Figure 5  Several metabolites have differential levels in paediatric PAH (vs non-PAH control) in SVC and PA. Mean±SEM statistical test: Mann-Whitney U test with FDR correction for multiple testing. Significance levels: *p<0.15, **p<0.05 for FDR-adjusted p values. (A) Plasma levels of heptanoate (7:0) in SVC (significant upregulation), PA and AAO. (B) Plasma levels of caproate (6:0) in SVC (significant upregulation), PA and AAO. (C) Plasma levels of glycocholenate sulfate in SVC (significant upregulation), PA and AAO. (D) Plasma levels of 1-stearoyl-GPI (18:0) in SVC, PA (significant upregulation) and AAO. (E) Plasma levels of N6-acetyllysine in SVC, PA (significant downregulation) and AAO. AA, AAO, ascending aorta; Con, control; FC, fold change; GPI, glycosylphosphatidylinositol; mPAP, mean pulmonary arterial pressure; mAAO, mean ascending aorta pressure; PA, pulmonary artery; PAH, pulmonary arterial hypertension; PVRi, pulmonary vascular resistance index; RVAW, right ventricular anterior wall diameter; RVEDD, right ventricular end-diastolic diameter; SVC, superior vena cava; WU, Wood units.
Figure 6 Disruption of β-oxidation in the right ventricle in human PAH indicates major disturbance of lipid and energy metabolism (model). Our results in this article are shown in blue font. Conversion of carboxylic acids to dicarboxylic acids is facilitated by ω-oxidation, which results in conversion of the methyl group (CH₃) at the ω-end into a carboxyl group (COOH). The produced dicarboxylic acids (eg, octadecanedioate) are further metabolised by β-oxidation. Unlike β-oxidation, ω-oxidation does not require acyl-CoAs and provides an alternative for disrupted β-oxidation. The accumulation of octadecanedioate (a dicarboxylic acid) in PAH that we demonstrated as trans-RV blood plasma gradient, strongly suggests disruption of peroxisomal β-oxidation in the hypertensive RV, because very long chain and long chain dicarboxylic-CoAs (such as the end-product of octadecanedioate ω-oxidation) can only be metabolised by peroxisomal β-oxidation. The accumulation of stearoylcarnitine and other acylcarnitines suggests incomplete mitochondrial fatty acid oxidation (FAO), probably resulting from FAO flux outpacing TCA flux. The lipotoxic effects of the molecules with detected accumulation in the PAH RV plasma concentration gradients include: cardiomycocyte apoptosis for octadecanedioate and activation of pro-inflammatory signalling for stearoylcarnitine. Both of these effects are hallmarks of heart failure. Additionally, disruption of β-oxidation results in lower ATP production and thereby impaired cardiac performance. CPT1, carnitine palmitoyltransferase I; ETC, electron transport chain; ER, endoplasmic reticulum; LCFA, long-chain fatty acid; PAH, pulmonary arterial hypertension; RV, right ventricle; TCA, tricarboxylic acid cycle; VLCFA, very-LCFA.

and lipid transpulmonary gradients and their correlation with haemodynamics is provided in the online supplement (supplementary results).

Metabolite plasma levels of FAs, bile acids, glycerophosphoinositols and amino acids are altered in PAH at the individual SVC and PA catheterisation sites and correlate with invasive haemodynamic and echocardiographic variables

In order to identify global differences in levels of plasma metabolites for each site, we performed comparisons between groups (PAH vs controls; that is, here subjects did not serve as their own controls). Five metabolites were significantly (FDR<0.15) higher in the SVC and three were differentially concentrated in the PA of PAH patients vs controls (figure 5, online supplement file 1). These metabolites are further discussed in the online supplement (supplementary results).

Using metabolite ratios did not improve correlations of metabolites with haemodynamic/echocardiographic variables

We explored the metabolite ratios that were identified based on significantly differentially concentrated metabolites (FDR<0.15) (numerator) and the corresponding metabolites located in a comprehensive network of human metabolites with a distance ≤2 (denominator), as described in the online supplement. Using the ratios did not produce significantly better correlations of metabolites with haemodynamic/echocardiographic variables.

Arachidonate is a hub in metabolite network of second-order correlations

Finally, we performed metabolite network analysis to identify ‘regulatory hub’ metabolites with the largest impact on the identified, differentially concentrated metabolites in each experimental setting (trans-RV, transpulmonary, or between-group comparisons in a single catheterisation site). Among the transpulmonary gradients, arachidonate (20:4n6) stood out as a ‘hub’ node with second-order correlations (r=0.40 to 5) other metabolites, however the correlations were only moderate (0.40≤r ≤0.49). The detailed results of the metabolite network analysis are presented in the online supplementary figure S2. A complete list of metabolites, metabolite ratios, lipid species and FAs with significantly differential concentration profiles in all experimental settings is in online supplementary file 1. Metabolite annotation and identifiers are presented in online supplementary file 2.

DISCUSSION

Here, we first present a translational metabolomics PAH study and demonstrate differential gradients of a number of metabolites across the hypertensive RV and across the pulmonary circulation in blood plasma of PAH versus non-PAH patients (FDR<0.15; online supplementary file 1). We provide the first evidence for a likely major disruption of peroxisomal β-FAO in the hypertensive RV in human PAH, by linking trans-RV accumulation of octadecanedioate to clinically relevant indicators of RV function. Additionally, the detrimental sequelae of dysfunctional peroxisomal β-fatty acid oxidation (FAO) extends to disrupted metabolism of very-long-chain FAs (including the subsequent mitochondrial β-FAO of the corresponding chain-shortened Acyl-CoAs), long-chain dicarboxylic acids, eicosanoids, docosahexaenoic acid (DHA), among other alterations. The mechanisms involved in these changes are, most likely, either altered uptake into the organ (lung or heart), or differential release from the organ. The most pronounced differential trans-RV and transpulmonary gradients are presented in the figures 2 and 4. The sections on: (1) accumulation of lysophospholipids across the right ventricle (trans-RV), (2) transpulmonary circulation metabolite gradients, (3) differential same-site metabolite concentration levels in paediatric PAH and (4) trans-RV PAH-specific lipids associated with CVD are presented in the online supplement. The results of our subsequent lipidomics study (Lipidyzer platform) further supported our interpretations and conclusions (online supplementary figures S4 and S6).

Evidence for a block of peroxisomal β-oxidation in the hypertensive RV

In homeostasis, FAs are primarily oxidised by β-oxidation in mitochondria (long-chain, medium-chain and short-chain FAs)
and by α-oxidation and β-oxidation in peroxisomes (very-long-chain FAs (VLCFAs), long-chain dicarboxylic FAs, eicosanoids). Additionally, 5%–10% of FAO occurs through ω-oxidation in the smooth endoplasmic reticulum (long-chain and very-long-chains FAs), thus producing long-chain dicarboxylic FAs.18 Acetyl-CoA, required for delivery of acetyl groups into the tricarboxylic acid (TCA) cycle, are directly produced by peroxisomal and/or mitochondrial β-oxidation of FAs, but not by ω-oxidation.17 18

However, when β-oxidation is disrupted, α-oxidation provides an alternative to prevent accumulation of the long-chain FAs (LCFAs) and the detrimental effects such FA accumulation may cause in pathological conditions, e.g., in PAH.18

Octadecanedioate belongs to the group of dicarboxylic FAs, which are generated by conversion of the terminal methyl group of a FA into a carboxyl group by ω-oxidation; dicarboxylic FAs are then further β-oxidised into shorter dicarboxylic acids (figure 6). As a long-chain dicarboxylic fatty acid, octadecanedioate is β-oxidised in peroxisomes rather than mitochondria.17 Intriguingly, in our translational study, octadecanedioate trans-RV gradients were present and had a step-up in PAH and a prominent step-down in the non-PAH controls (Con) (figure 2C), indicating dysfunctional peroxisomal β-oxidation in PAH versus controls. The latter conclusion is also supported by the observed accumulation of other dicarboxylic acids (dodecanedioate and tetradecanedioate) trans-RV (PAH vs Con), though not reaching the significance threshold (online supplementary table S7). Additionally, the trans-RV Lipidyzer (lipidomics) analysis revealed an accumulation of VLCFAs (carbon chain length ≥22) that are oxidised exclusively by peroxisomal β-oxidation (or much less efficiently by ω-oxidation). In particular, lipid species TAG54:10–FA22:6 with the C22:6 acyl group (DHA) had a significant step-up in PAH and a step-down in controls (online supplementary table S7). In addition, the DAG component of DHA, that is, DAG(FA22:6) also had a step-up in PAH and a small step-down in controls (online supplementary table S7). This cumulative evidence further supports our conclusion about a likely peroxisomal β-oxidation block in the hypertensive RV (online supplementary figure S4 and S6).

Importantly, octadecanedioate trans-RV gradients correlated with clinically relevant variables of RV systolic function and PAH severity, such as TAPSE (figure 2C), RVAWD, mPAP and PVRi (table 2). Consistently, octadecanedioate was previously reported to be upregulated in both peripheral venous blood13 and explant lung tissue from PAH patients.19

### Table 2 Differential trans-RV metabolite concentration gradients and their correlation with invasive haemodynamic/echocardiographic variables

| Metabolite or metabolite ratio | Fold change Control | Fold change PAH | FDR-adjusted p value | Selected haemodynamic or echocardiographic variables | r | P value |
|-------------------------------|---------------------|-----------------|----------------------|------------------------------------------------------|---|--------|
| Subclass: GPE Role: Phospholipid biosynthesis, glycerophospholipid metabolism, lipid metabolism | 1-palmitoyl-GPE (16:0) | 0.70 | 2.31 | 0.0134 | TAPSE, cm | −0.73 | 0.0254 |
| | sPAP, mm Hg | 0.55 | 0.0424 |
| Subclass: GPE Role: Phospholipid metabolism, lipid transport, lipid metabolism, fatty acid metabolism | 1-oleoyl-GPE (18:1) | 0.78 | 1.48 | 0.0767 | TAPSE, cm | −0.83 | 0.0104 |
| | sPAP | −0.52 | 0.0533 |
| Subclass: Fatty acids and conjugates Role: Lipid transport, lipid metabolism, fatty acid metabolism | Octadecanedioate | 0.77 | 1.09 | 0.0157 | TAPSE, cm | −0.72 | 0.0444 |
| | RVAWD, cm | 0.52 | 0.0560 |
| | Tricuspid valve E/A | −0.71 | 0.0226 |
| | mPAP, mm Hg | 0.56 | 0.0371 |
| | PVRI, WU·m−2 | 0.54 | 0.0565 |
| Subclass: Fatty acid esters Role: Lipid transport, Lipid metabolism, Fatty acid metabolism | Stearoyl carnitine | 0.74 | 1.21 | 0.0582 | sPAP, mm Hg | 0.52 | 0.0814 |

For gradient analysis, the p values were generated using Wald χ² test (FDR<0.15). A more comprehensive data set, including metabolite ratios, is shown in online supplementary table S4.

FDR, false discovery rate; GPE, glycerophosphoethanolamine; mPAP, mean pulmonary arterial pressure; PAH, pulmonary arterial hypertension; PVRI, pulmonary vascular resistance index; RV, right ventricle; RVAWD, right ventricular anterior wall diameter; sPAP, systolic pulmonary arterial pressure; TAPSE, tricuspid annular plane systolic excursion; WU, Wood units.

Trans-RV accumulation of metabolites known to drive lipotoxicity in the heart

Aside from the peroxisomal β-oxidation block discussed above, accumulation of octadecanedioate can be attributed to increased LCFAs concentrations (including octadecanoic acid (18:0)) due to impaired mitochondrial β-oxidation (manifested via accumulation of acylcarnitines in our dataset). In the Lipidyzer experiments LCFAs accumulated trans-RV (figures 3A and online supplementary table S4). Importantly, accumulated LCFAs, particularly in their saturated form, are considered to be a potent driver of cardiac lipotoxicity,20 for example in neonatal rat ventricular myocytes.21 Additionally, cardiomyopathy is associated with elevated serum levels of TAGs (triglycerides) and non-esterified fatty acids in obesity and type 2 diabetes, that is, diseases which are characterised by accumulation of free fatty acids and neutral lipids within cardiomyocytes.22 It has been reported that lipid overload causes cardiac lipotoxicity leading to cellular dysfunction, cell death and organ dysfunction.22 Cardio-myocyte apoptosis not only decreases RV contractility in PAH, but is a hallmark of heart failure in general,23 besides fibrosis and inflammation. Indeed, several studies provided strong evidence for cardiac lipotoxicity as a metabolic component of PAH-related RV dysfunction in animal models4 24 and PAH patients.25
Incomplete mitochondrial β-oxidation in the hypertensive RV in PAH

In the current study, we found a step-up in trans-RV stearoylcarnitine gradients in PAH and a step-down in controls (figure 2E), suggesting incomplete mitochondrial β-oxidation (ie, lack of reconversion of stearoylcarnitine into Acyl-CoAs) in PAH. The net result is an accumulation of long-chain acylcarnitines due to incomplete β-oxidation still outpacing the TCA cycle flux—a biochemical condition also found in insulin resistance.26 Thus, we conclude that the trans-RV accumulation of stearoylcarnitine in our study is due to incomplete mitochondrial β-oxidation and decreased TCA flux in response to the metabolic switch from glucose and lipid oxidation toward glycolysis. Several other acylcarnitines accumulated trans-RV, shown in grey in online supplementary figure S4, however not reaching the pre-selected FDR significance cut-off.

In human PAH-cardiomyocytes, glucose uptake and glycolysis are increased, mitochondrial β-oxidation is decreased (resulting in the smaller net amount of acylcarnitines vs control25), and TCA flux is suppressed even more (probably due to the simultaneous reduction in glucose oxidation). We propose that accumulating, unused acylcarnitines (online supplementary figure S4), such as stearoylcarnitine, are then gradually exported into the blood stream of PAH patients. Moreover, long-chain acylcarnitines have been linked to activation of pro-inflammatory pathways in a macrophage cell line27 thereby potentially contributing to the inflammatory component of heart failure.

Importantly, our lipidomics experiments (Lipidyzer platform) provided additional evidence for incomplete mitochondrial β-oxidation in the hypertensive RV. While Lipidyzer cannot measure accumulation of acylcarnitines that would directly prove this point, we still observed accumulation of LCFAs. In animals LCFAs are preferentially oxidised via mitochondrial β-oxidation, while peroxisomes preferentially β-oxidise FAs not meeting the substrate range of the mitochondria, that is, VLCFAs and branched-chain FAs.28 Therefore, the accumulation of LCFAs observed in our lipidomics experiments further supports our conclusion about compromised mitochondrial β-oxidation in the hypertensive RV (online supplementary figure S4).

Cardiomyocyte FAO in PAH

We previously linked insulin resistance and dyslipidemia to PAH in mice and adult PAH patients.29 30 More recently, we demonstrated that the peroxisome proliferator-activated receptor gamma (PPARγ) agonist pioglitazone reverses PAH and prevents RV failure in the Sugen-hypoxia (SuHx) rat model, by boosting FAO in cardiomyocytes.3 Our current results on disturbed β-FAO in the RV of children with PAH are in line with our previous findings on (1) impaired FAO and mitochondrial disarray in RV tissue from end-stage PAH patients, (2) impaired FAO in the hypertensive RV of SuHx rats and (3) the normotensive RV of mice with targeted deletion of PPARγ in cardiomyocytes.3 Impaired β-FAO in mitochondria and peroxisomes will result in decreased ATP production and thus decreased contractile performance in stressed cardiomyocytes (figure 6).5 However, decreased β-FAO (and induced glycolysis) goes along with decreased oxygen consumption; the latter could be a useful rescue mechanism in end-stage PAH with severe RVH, largely elevated right-ventricular end-diastolic pressure (RVEDP), decreased coronary perfusion, and consequently limited myocardial oxygen supply.

Key questions

What is already known on this subject?

► Metabolic dysfunction occurs in several PAH animal models, however, its role in human disease is not well characterised. We hypothesised that trans-right ventricle (RV) and transpulmonary metabolite concentration gradients exist in PAH, and might correlate with prognostic invasive haemodynamic and echocardiographic variables.

What might this study add?

► We performed combined right and left heart catheterisation in PAH patients without shunt, and identified—for the first time—such trans-RV and transpulmonary metabolite gradients. We demonstrate accumulation of dicarboxylic acids (eg, octadecanediolate) and acylcarnitines (eg, stearoylcarnitine), among other metabolites, indicating a block in β-fatty acid oxidation (β-FAO) in the hypertensive right ventricle.

How might this impact on clinical practice?

► The identified trans-RV and transpulmonary metabolite gradients not only indicate a major block in β-FAO trans-RV, but also correlate with haemodynamic determinants of clinical outcome, and can become emerging biomarkers and therapeutic targets in PAH.

Strengths and limitations

We followed strict enrollment criteria for paediatric patients undergoing invasive catheterisation (see methods). As a result, we have relatively small sample sizes. To increase robustness of our data, we excluded measurements with a missing value in at least one of the three catheterisation sites (due to very low concentrations or technical issues). Therefore, we could only unravel the metabolites with large enough concentration gradient difference (figures 2–4) or concentration difference (eg, PAH SVC vs Con SVC, figure 5) (ie, sufficient effect size) to still produce significantly low p values. See the online supplement for more details.

Conclusions

Taken together, we identified for the first time in human disease (PAH) trans-RV and transpulmonary metabolite concentration gradients in blood plasma, which may be involved in (anti-)remodelling processes in the RV and pulmonary vessels. The identified alterations in trans-RV gradients likely indicate (1) a major block in peroxisomal/mitochondrial β-FAO and (2) emergence of lipotoxicity in the hypertensive RV of PAH patients as potential cause for subsequent RV failure. The clinical importance of the differential metabolite levels presented here is supported by their correlations with key haemodynamic and echocardiographic variables used for diagnosis, risk assessment and selection of therapy in clinical PAH. By doing so, we unravelled new metabolic biomarkers and emerging targets for future PAH therapy.
Pulmonary vascular disease

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Correction notice Since this paper was first published online, the ORCID IDs have been added to authors Martin Giera, Harald Bertram and Georg Hansmann

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Contributors PC performed data analysis and wrote the manuscript. MG supervised lipodomics analysis and edited the manuscript for important intellectual content. GK performed initial data analysis and edited the manuscript for important intellectual content. AA performed the metabolomics measurements, quality control, prepared data for metabolite annotation and edited the manuscript for important intellectual content. GH designed the study, obtained RB approval, blood samples, written consent and funding, performed cardiac catheterisations and data analysis and wrote the manuscript.

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Ethics approval Human ETDA plasma samples were handled anonymously, according to the principles expressed in the Declaration of Helsinki. The study was approved by the ethics committee of Hannover Medical School (IRB #2200).

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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SUPPLEMENTAL MATERIALS

Trans-right-ventricle and transpulmonary metabolite gradients in human pulmonary arterial hypertension

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SUPPLEMENTARY INTRODUCTION

Invasive assessment of right and left heart hemodynamics is mandatory for diagnosis of pulmonary hypertension (PH) and its classification (PAH is WHO group 1 PH). [1, 2] In addition, transthoracic echocardiography is a readily available, easy to use method to evaluate RVH, dilation and systolic function non-invasively.[3] Several invasive-hemodynamic, echocardiographic and clinical variables can be used in combination to estimate patient risk and prognosis, and to determine the best PAH therapy.[2, 4] However, due to heterogeneous etiologies of PAH and confounding factors such as variable preload (volume status) and inter-observer variability, reliance on non-invasive hemodynamic variables alone is problematic.[1]

Sole right heart catheterization is frequently performed at PH centers, so that, most of the above studies used either peripheral arterial blood[5] or even pulmonary arterial wedge specimen[6, 7, 8] as "post lung samples". Due to the very high variability of such circulating "remote post lung markers", statistical power, result interpretation, and conclusions were limited.

A few studies identified venous metabolites as potential biomarkers for PAH[9, 10] or CVD-related mortality.[11] In the largest study, investigators measured plasma concentrations of 1416 circulating metabolites in peripheral venous blood, and found that 53 of those metabolites distinguished adult PAH patients from healthy controls.[9] In a separate study, plasma samples obtained by right heart catheterization and radionuclide ventriculography from PAH patients were screened for 105 metabolites by targeted mass spectrometry: 21 were identified as indicators of right ventricular-pulmonary vascular dysfunction.[10]
SUPPLEMENTARY METHODS

1. Cardiac Catheterization

For the purpose of this prospective study, pediatric PAH was defined as per 2015/2016 international guidelines: mean pulmonary artery pressure (mPAP) ≥25 mmHg, a pulmonary arterial wedge pressure (PAWP) ≤15 mm Hg, and pulmonary vascular resistance index (PVRi) >3 WU·m² when >3 months old, at sea level. PAH and non-PAH patients of both genders, more than 3 months and less than 18 years old, were enrolled and underwent combined right and left heart catheterization.

The most recent World Symposium on PH (WSPH) in Nice (2018) decreased the cut-off value for mean pulmonary arterial pressure, so that PAH was defined as mPAP > 20 mmHg at rest. PAH is a subgroup of PH in which pre-capillary PH is dominating, defined as combination of a mPAP > 20 mmHg, a pulmonary arterial wedge pressure (PAWP) ≤15 mm Hg (alternatively: LV end-diastolic pressure or mean left atrial pressure), and a pulmonary vascular resistance (PVR) >3 Wood units (WU) (in children: PVR index >3 WU·m² when >3 months old, at sea level).

All patients underwent right and left heart catheterization in room air, under conscious intravenous sedation (propofol) and local anesthesia for femoral access (2 venous sheaths, 1 arterial sheath), with the exception of patients 7 and 8 in the Metabolon study (6.6 kg and 4.5 kg) who had Trisomy 21 and had to be intubated and mechanically ventilated. Three catheters were positioned in the SVC, right or left pulmonary artery (wedge catheter), and the ascending aorta (pigtail catheter; Fig.1). Arterial blood gas analysis confirmed normoventilation. Once these catheter positions were stably achieved, pressure recordings, blood gas analyses and EDTA blood samples were obtained near-simultaneously within one minute. Pulmonary blood flow and cardiac index were calculated by applying the Fick principle.
2. Transthoracic echocardiography

All patients underwent transthoracic echocardiography (iE33, Philips) on the day preceding the cardiac catheterization following a standardized protocol. [3]

3. Blood plasma collection

EDTA blood was spun down at 1300g and room temperature for 10 minutes, within 20 minutes after sample collection. Plasma was then aliquoted in 500 µl aliquots and immediately frozen at -80°C. After thawing, plasma samples underwent a high speed spin step at 18,000g, 4°C for 15 minutes.

4. Non-targeted metabolite measurements

Plasma samples were stored at -80 °C prior to analysis at Helmholtz Zentrum München, Germany. On the day of extraction, samples were thawed on ice, were randomized, and were distributed into 3 batches. A hundred µL of the plasma were pipetted into a 2 mL 96-well plate. In addition to samples from this study, a pooled human reference plasma sample (Seralab, West Sussex, UK) were extracted as samples of the study and placed 7 wells of each batch. These samples served as technical replicates throughout the data set to assess process variability. Besides those samples, 100 µL of water was extracted as samples of the study and placed in 6 wells of each 96-well plate to serve as process blanks.

Protein was precipitated and the metabolites in the plasma samples were extracted with 475 µL methanol, containing four recovery standard compounds to monitor the extraction efficiency. After centrifugation, the supernatant was split into 4 aliquots of 100 µL each onto two 96-well microplates. The first 2 aliquots were used for LC-MS/MS analysis in positive and negative electrospray ionization mode. Two further aliquots on the second plate were kept as
Chouvarine P, ..., Hansmann G (Jan. 2020) Trans-RV and transpulmonary microRNA gradients in human PAH

... a reserve. The samples were dried on a TurboVap 96 (Zymark, Sotax, Lörrach, Germany). Prior to LC-MS/MS in positive ion mode, the samples were reconstituted with 50 µL of 0.1% formic acid and those analyzed in negative ion mode with 50 µL of 6.5 mM ammonium bicarbonate, pH 8.0. Reconstitution solvents for both ionization modes contained further internal standards that allowed monitoring of instrument performance and also served as retention reference markers. To minimize human error, liquid handling was performed on a Hamilton Microlab STAR robot (Hamilton Bonaduz AG, Bonaduz, Switzerland).

LC-MS/MS analysis was performed on a linear ion trap LTQ XL mass spectrometer (Thermo Fisher Scientific GmbH, Dreieich, Germany) coupled with a Waters Acquity UPLC system (Waters GmbH, Eschborn, Germany). Two separate columns (2.1 x 100 mm Waters BEH C18 1.7 µm particle) were used for acidic (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in methanol) and for basic (A: 6.5 mM ammonium bicarbonate pH 8.0, B: 6.5 mM ammonium bicarbonate in 95% methanol) mobile phase conditions, optimized for positive and negative electrospray ionization, respectively. After injection of the sample extracts, the columns were developed in a gradient of 99.5% A to 98% B in 11 min run time at 350 µL/min flow rate. The eluent flow was directly connected to the ESI source of the LTQ XL mass spectrometer. Full scan mass spectra (80 – 1000 m/z) and data dependent MS/MS scans with dynamic exclusion were recorded in turns. Metabolites were annotated by curation of the LC-MS/MS data against proprietary Metabolon’s chemical database library (Metabolon, Inc., Durham, NC, USA) based on retention index, precursor mass and MS/MS spectra. In this study, 427 metabolites, 289 compounds of known identity (named biochemical) and 138 compounds of unknown structural identity (unnamed biochemical) were identified. The unknown chemicals are indicated by a letter X followed by a number as the compound identifier. The metabolites were assigned to cellular pathways based on PubChem, KEGG, and the Human Metabolome Database.
5. Untargeted lipidomics

Lipidomics analysis was carried out on the commercial Lipidyzer platform, according to the manufacturer’s instructions (Sciex). Lipid analysis was performed after methyl-tert.-butyl ether extraction in flow-injection mode, separating lipid classes by differential mobility spectroscopy, followed by tandem mass spectrometry of lipid species with a QTrap 5500 operated in multiple reaction monitoring mode. Lipid species were identified and quantified on the basis of characteristic mass spectrometric transitions. Commercial Lipidyzer software automatically calculated lipid species concentrations. All samples were analyzed in a randomized fashion. Control plasma samples, as well as fortified plasma samples, were assessed daily as quality controls. Relative standard deviations of quality control samples were below 15% for all lipid classes, except for sphingomyelin, for which a relative standard deviation of 25% was noted. In summary, the Lipidyzer is a validated quantitative lipidomics platform allowing the selective and quantitative analysis of 836 individual lipid species. It allows an unprecedented detailed view of the plasma lipidome in our study cohort, thereby providing highly orthogonal and complementary information to the Metabolon platform used in our clinical investigation.

6. Statistical analysis (Metabolon study, metabolomics)

Metabolites with a missing value in at least one of the three catheterization sites were removed from the gradient analysis. For the trans-RV and transpulmonary gradient analysis we used mixed effects models with log2 of fold change (FC) between two catheterization sites (PA vs. SVC for the trans-RV gradient and AAO vs. PA for the trans-pulmonary gradient) as the dependent variable, groups (PAH or Control) as an independent variable, and each patient as a random effect (log2(FC)~Group, random=~1|Patient). To avoid technical artifacts, iterative outlier removal procedure for the outliers identified using Grubb’s test (p-val>0.1) was applied to log2 of the relative ion count values at each of the two
catheterization sites prior to modeling. Following the outlier removal, metabolites with less than three control or four PAH FCs were removed. The generated models were evaluated using the Anova function from the car R package, and the p-values generated by Type II Wald chi-square test (using the “car” R package[21]) were further corrected for multiple testing following the modified FDR procedure described in Li et al.[22] The three between group comparisons at each catheterization site were performed using Mann-Whitney U test after the iterative outlier removal procedure for the outliers identified using Grubb’s test (p-val>0.1) was applied to the values of a given catheterization site. The same modified FDR multiple testing correction[22] was performed.

For each experimental setting described above we performed principal component analysis (PCA) aiming to show how well all metabolites in our analyses are able to discriminate phenotype (PAH vs. non-PAH). The PCA was performed on the preprocessed data for each comparison (as described above). The results showing the first two principal components are presented in Fig. S1.

Metabolite ratios were created such that numerator contained significantly differentially concentrated metabolites (FDR<0.15) and all respective denominators contained neighboring nodes with distance ≤ 2 from a network of human metabolites (a combination of knowledge-based Recon network and data-driven Metabolon network). The resulting ratios underwent the same analysis as single metabolites. In most cases the ratios had smaller differences (effect sizes) compared to the respective single metabolite. However, in the case of the 1-stearoyl-GPI (18:0) / 10-heptadecenoate (17:1n7) ratio in the PA, the fold change increased to 4.52, compared to 3.85 for 1-stearoyl-GPI (18:0) alone. Besides this exception, the above ratio analysis in our study did not produce significantly better correlations of metabolites with hemodynamic/echocardiographic variables.

Metabolites and ratios with low adjusted p-values were correlated with hemodynamics. The trans-RV and trans-pulmonary gradient fold changes (log2(FC)) were
correlated using Pearson's r. The relative ion counts of the significantly differentially expressed (PAH vs Control) metabolites within one of the catheterization sites were correlated with hemodynamic values using Spearman's rho.

Network analysis presented below was performed by calculating second-order Pearson's correlations for the fold changes in the gradient analysis and Spearman's correlations for the normalized ion counts in the between group analysis in the SVC and PA. The networks were visualized using the MetScape Cytoscape application.
Figure S1. Principal component analysis of metabolite variability in each experimental setting: (A) trans-RV gradient, (B) transpulmonary gradient, (C) superior vena cava, (D) pulmonary artery, (E) ascending aorta.
Metabolite concentration correlation network analysis

To identify regulatory hubs with the most impact on the identified differentially concentrated metabolites in each experimental setting (trans-RV, transpulmonary, or between group comparisons in a single catheterization site), we performed network analysis based on the second-order correlations among the differentially concentrated metabolites with a p-value < 0.05 (prior to the FDR adjustment), as described above. The results of the analysis revealed that in the trans-RV, none of the gradients formed a noticeable “hub” node that would exert influence on multiple neighboring metabolites in this correlation-driven network (Fig. S2 A). The highest degree nodes in the trans-RV setting represented 1-palmytoyl GPE (16:0), pyroglutamine, and arachidate (20:0), each of which correlated with three other metabolites \((r \geq 0.40)\). Among the transpulmonary gradients, arachidonate (20:4n6) stood out as a “hub” node with second-order correlations \((r \geq 0.40)\) to five other metabolites, however the correlations were only moderate \((0.40 \leq r \leq 0.49)\) (Fig. S2 B). This influence of arachidonic acid (arachidonate (20:4n6)) is expected, given the central role it plays in eicosanoid metabolism (further discussed in the “Discussion of selected metabolites with differential transpulmonary gradients (Trans-PC gradients)” section below). In the single site comparisons the noticeable “hub” metabolites were taurodeoxycholate (4 correlations, \(0.42 \leq \rho \leq 0.50\)) and heptanoate (7:0) (4 correlations, \(0.40 \leq \rho \leq 0.51\)) in the SVC (Fig. S2 C), and 1-(1-ethyl-palmitoyl)-GPE (P-16:0) (4 correlations, \(0.43 \leq \rho \leq 0.51\) and \(\rho = -0.50\)) in the PA (Fig. S2 D).
Figure S2. Metabolites exerting the most influence are identified as “hub” nodes using network analysis based on the second-order correlations among the differentially concentrated metabolites (PAH vs. controls; p-val<0.05, without FDR correction). Positive correlations are shown in red, negative correlations are shown in blue. The line thickness indicates correlation strength. Metabolites without a single correlation (r ≤ -0.4 or r ≥ 0.4; rho ≤ -0.4 or rho ≥ 0.4, for single sites, i.e. C and D) were excluded. (A) Network of differential trans-RV gradients based on fold changes (PA vs. SVC) of 32 metabolites. (B) Network of differential transpulmonary gradients based on fold changes (AAO vs. PA) of 26 metabolites. (C) Network of differentially concentrated metabolites in the SVC based on normalized ion counts of 24 metabolites. (D) Network of differentially concentrated metabolites in the PA based on normalized ion counts of 20 metabolites.
7. Statistical analysis (Lipidyzer study; lipidomics)

We followed the same statistical analysis methodology for analysis of lipids species and fatty acid (FA) concentrations obtained from the Lipidyzer platform. The only exception was not using the log2 transformation in the outlier removal procedure, since the raw data was normally distributed. We also did not perform separate ratio analysis, since the ratios were already included in the output. Correlation network analysis was also omitted, since the Lipidyzer experiments were meant to be used as support for metabolite profiling study described above.

For each experimental setting we performed principal component analysis (PCA) aiming to show how well all lipid species and FAs in our analyses are able to discriminate the phenotypes (PAH vs. non-PAH). The PCA was performed on the preprocessed data for each comparison (as described above). The results showing the first two principal components are presented in Fig. S3.
Figure S3 Principal component analysis of lipid species and fatty acid variability in each experimental setting (Lipidizer experiments): (A) trans-RV gradient, (B) transpulmonary gradient, (C) superior vena cava, (D) pulmonary artery, (E) ascending aorta.
SUPPLEMENTAL RESULTS

Differential transpulmonary gradients (PAH vs. control) of several circulating metabolites exist and correlate with invasive hemodynamic and echocardiographic variables

We identified seven metabolites with significantly (FDR<0.15) different levels (PAH vs. control) across the pulmonary circulation (AAO vs. PA). The transpulmonary plasma concentration gradients of four of these metabolites, i.e., N6-acetyllysine (step up in PAH), 2-palmitoyl-GPC (16:0) (step down in PAH), N-acetylcarnosine (step down in PAH), and azelate (nonanedioate) (step down in PAH) along with their correlation to hemodynamic and echocardiographic variables are shown in Fig. 4 (presented metabolites were selected based on function and effect size). These metabolites belong to amino acids (N6-acetyllysine), glycerophosphocholines (2-palmitoyl-GPC (16:0)), hybrid peptides (N-acetylcarnosine), and the group of fatty acids and their conjugates (azelate). Correlation analysis of the transpulmonary log2 fold changes of these four circulating metabolites with the key hemodynamics revealed that they had moderate to strong, positive or negative correlations with TAPSE, RVAWD, mPAP, mTPG, dTPG, mPAP/mAAO, PVRi, and other key variables (Table S5). Plasma concentration gradients of N6-acetyllysine positively correlated positively with hemodynamic indicators of PVD severity (mPAP, mTPG, dTPG, PVRi) and RV hypertrophy (RVAWD), while the levels of 2-palmitoyl-GPC (16:0) had negative correlations with the indicators of PVD severity (mRAP, mPAP, mPAP/mAAO, PVRi), hypertrophy (RVAWD), and advanced PAH with RV dilation (RVEDD). Azelate (nonanedioate) negatively correlated with fewer of the PVD severity biomarkers (mPAP, dTPG). N-acetylcarnosine had fewer correlations with the established hemodynamic biomarkers, but had the best correlation with PAWP, which is a surrogate for LVEDP and thus LV diastolic function (a LVEDP > 15 mm Hg indicates postcapillary PH in HFpEF).[23] as opposed to pure pre-capillary PH in PAH where LVEDP is 15mmHg or less).
Finally, the lipidomics analysis of trans-pulmonary circulation revealed that phosphatidylcholines PC(FA20:5) and PC(18:0/20:5), diacylglycerol DAG(FA18:2), free fatty acid FFA(FA18:4), triacylglycerol TAG(FA20:5), and cholesterol ester CE(FA20:2) had a step-down in PAH and a step-up in controls (Fig. 3B).

Metabolite plasma levels of fatty acids, bile acids, glycerophosphoinositols, and amino acids are altered in PAH at the individual SVC and PA catheterization sites and correlate with invasive hemodynamic and echocardiographic variables

In order to identify global differences in levels of plasma metabolites for each site, we performed comparisons between groups (PAH vs. controls; i.e., here subjects did not serve as their own controls). Five metabolites were significantly (FDR<0.15) higher in the SVC of PAH patients vs. controls. Three of these metabolites (Fig. 5 A,B,C), belong to either fatty acids and conjugates (heptanoate (7:0) and caproate (6:0)) or the group of bile acids, alcohols and derivatives (glycocholenate sulfate).

Heptanoate (7:0) and glycocholenate sulfate concentrations exhibited positive correlations with variables of PVD severity and RV hypertrophy (Table S6). Caproate also positively correlated with hemodynamic variables of PVD severity, and negatively correlated with the variables related to the LV (due to the increased LV compression in PAH) (Table S6).

In the PA, three metabolites were significantly (FDR<0.15) differentially concentrated, of which 1-stearoyl-GPI (18:0) and N6-acetyllysine had likely biologically relevant effect sizes (Fig. 5 D,E). 1-stearoyl-GPI (18:0), which belongs to glycerophosphoinositols, was upregulated and had moderate correlations with the PVD severity hemodynamic variables and a moderate negative correlation with the longitudinal systolic RV function variable, TAPSE (Table S6). N6-acetyllysine, an acetylated amino acid, was downregulated in PAH
and correlated negatively with the hemodynamic variables of PVD severity, hypertrophy, and advanced PAH/RV pressure overload.
SUPPLEMENTAL DISCUSSION

Trans-RV accumulation of metabolites known to drive lipotoxicity in the heart

In the Lipidyzer experiments the following LCFAs were accumulated: C14 (TAG50:5-FA14:0), C16 (TAG52:6-FA16:1 and TAG51:3-FA16:1), C18 (TAG55:3-FA18:2, TAG54:4-FA18:0, and TAG56:4-FA18:2), and C20 (CE20:2) (Fig. 3A and 6). Octadecanoic acid was not included in the Metabolon platform. However, we identified trans-RV accumulation of LCFAs represented in the Metabolon platform, namely, eicosenoate (20:1) and arachidate (20:0) (Table S7); however, likely due to the low sample size the arachidate FDR-adjusted p-value was above the significance threshold. Importantly, accumulated LCFAs, particularly in their saturated form, are considered to be a potent driver of lipotoxicity,[24] for example in neonatal rat ventricular myocytes.[25]

Incomplete mitochondrial β-oxidation in the hypertensive RV in PAH

Long-chain acyl-CoAs are converted to acylcarnitines by carnitine palmitoyltransferase 1 (CPT1) located at the outer mitochondrial membrane.[26] The acylcarnitines are transported into the mitochondrial matrix by the mitochondrial inner membrane transporter carnitine acylcarnitine translocase (CACT), where they are reconverted back to free carnitine and long-chain acyl-CoAs by the enzyme CPT2.[26] In the current study, we found a step-up in trans-RV stearoylcarnitine gradients in PAH and a step-down in controls (Fig. 2E), suggesting incomplete mitochondrial β-oxidation (i.e., lack of reconversion of stearoylcarnitine into Acyl-CoAs) in PAH. The net result is an accumulation of long-chain acylcarnitines due to incomplete β-oxidation still outpacing the tricarboxylic acid (TCA) cycle flux – a biochemical condition also found in insulin resistance.[26] A microarray mRNA expression study on postmortem RV PAH-tissue found no significant difference between CPT1, CPT2, and genes responsible for transport of acylcarnitines into mitochondria (CACT, SLC22A5) and mitochondrial β-oxidation (Acyl-CoA dehydrogenase long chain; ACADL).[27] Thus, we conclude that the trans-RV accumulation of stearoylcarnitine in our study is due to
incomplete mitochondrial β-oxidation and decreased TCA flux in response to the metabolic switch from glucose and lipid oxidation toward glycolysis (Gly).[28] Several other acylcarnitines accumulated trans-RV, shown in gray in Fig. S4, however not reaching the pre-selected FDR significance cutoff.

Importantly, our lipidomics experiments (Lipidyzer platform) provided additional evidence for incomplete mitochondrial β-oxidation in the hypertensive RV. While Lipidyzer cannot measure accumulation of acylcarnitines that would directly prove this point, we still observed accumulation of LCFAs. In animals LCFAs are preferentially oxidized via mitochondrial β-oxidation, while peroxisomes preferentially β-oxidize FAs not meeting the substrate range of the mitochondria, i.e., VLCFAs and branched-chain FAs.[29] There is very little complementarity between the two beta-oxidation systems: Upregulation of one β-oxidation system via pharmacological induction of peroxisome proliferator-activated receptor α (PPARα) does not compensate for loss of function of the other beta-oxidation system.[29] Therefore, the accumulation of C14 (TAG50:5-FA14:0), C16 (TAG52:6-FA16:1 and TAG51:3-FA16:1), C18 (TAG55:3-FA18:2, TAG54:4-FA18:0, and TAG56:4-FA18:2), and C20 (CE20:2) LCFAs observed in our lipidomics experiments further supports our conclusion about compromised mitochondrial β-oxidation in the hypertensive RV (Fig S4).
Figure S4 Metabolomics and lipidomics analyses confirm disruption of peroxisomal and mitochondrial β-oxidation and lipotoxicity in the hypertensive RV of PAH patients. Two separate studies of slightly different cohorts of PAH patients and non-PAH controls confirm the same pathological processes. Here we show metabolites used as evidence in our conclusion of: i) peroxisomal β-oxidation block (accumulation of dicarboxylic acids and very-long-chain fatty acids); ii) incomplete mitochondrial β-oxidation (accumulation of acylcarnitines and long-chain fatty acids typically oxidized via mitochondrial β-oxidation); and iii) lipotoxicity (accumulation of long-chain fatty acids). The metabolites shown in gray font had FDR-adjusted p-values greater than the preselected cutoff (0.15). The arrows show the direction of change in concentrations (PA vs. SVC). Abbreviations: DAG, diacylglycerol; TAG, triacylglycerol; CE, cholesterol ester; FDR, false discovery rate.
Accumulation of lysophospholipids across the right ventricle (Trans-RV)

1-palmitoyl-GPE (16:0) is a lysophospholipid involved in phospholipid biosynthesis by producing glycerylphosphorylethanolamine, which is known to act as a growth stimulant in hepatocytes.[30] We found a significant trans-RV step-up in PAH and a step-down in controls of 1-palmitoyl-GPE (16:0) (Fig. 2A) that likely indicate increased release from the hypertensive, hypertrophied RV in PAH. The trans-RV step up of 1-palmitoyl-GPE (16:0) (2.3 fold) was pronounced and probably represents boosted production within cardiac myocytes and fibroblasts, followed by release into the blood stream. We speculate that the observed accumulation (differential metabolism) of lysophospholipids (1-oleoyl-GPE 18:1 also had a step-up of 1.48 fold; Table S4) is indicative of a dominant phospholipid catabolism perhaps in conjunction with impaired biosynthesis of phospholipids in the hypertensive RV. Interestingly, it has been shown that lysophospholipids accumulate in ischemic myocardium of dog hearts in conditions of no collateral flow or inflammatory cell infiltration.[31] Capillary rarefaction and decreased right coronary artery perfusion pressure are the factors that trigger ischemic conditions in the hypertrophied RV of PAH patients.[32]

Trans-RV PAH-specific lipid TAG54:4-FA18:0 is associated with incidence of cardiovascular disease (CVD)

Based on 685 plasma samples from the prospective population-based Bruneck study (2000–2010, n=90 events), several lipid species were associated with CVD-related mortality (myocardial infarction, ischemic stroke, and sudden cardiac death).[33] Importantly, we found one of the CVD-associated lipids TAG54:4-FA18:0 in our trans-RV measurements (step-up in PAH, step-down in controls). A few other CVD-associated lipids varied only in the number of double bonds with the following lipids that we identified in our trans-RV experiments: TAG52:6-FA16:1, TAG50:5-FA14:0, and CE20:2.
Transpulmonary circulation (trans-PC) metabolite gradients in pediatric pulmonary arterial hypertension

In the transpulmonary plasma measurements, differential gradients of several metabolites were identified for the first time, most notably a trans-PC step-down for Nε-acetyllysine and azelate (Fig. 4A and 4D). In contrast, the phosphatidylcholines PC(FA20:5) and PC(18:0/20:5) decreased in PAH but increased in controls trans-PC (Fig. 2B).

Nε-acetyllysine is an amino acid involved in lysine acetylation, which weakens histone-DNA or nucleosome-nucleosome interactions, causing conformational changes and destabilization of the nucleosome.[34] Across the pulmonary circulation (AAO vs. PA), Nε-acetyllysine had a step-up in PAH and a step-down in controls (Fig. 4A). Increased serum levels of Nε-acetyllysine have been reported to be associated with CVD-related mortality.[11] Moreover, Nε-acetyllysine has been shown to be upregulated in monocrotalin-exposed rat lung tissue (PH animal model)[35]. Thus, the likely mechanism of transpulmonary step-up of Nε-acetyllysine levels in our PAH patients is an increased release from the rather than the reduced uptake into the lung.

Azelate (nonanedioate), a C9 dicarboxylic acid, is involved in lipid metabolism, lipid transport, and serves as an energy source.[34] PAH patients had 48% higher azelate concentrations in venous blood plasma than controls.[36] Moreover, azelate levels positively correlated with the glycolytic rate in murine hearts[37], a mechanism that may also be particularly relevant in PAH vascular smooth muscle cells (SMCs). Previously, we unraveled in human pulmonary arterial SMCs (HPASMCs) the opposing roles of transforming growth factor beta (TGFβ1) and peroxisome proliferator-activated receptor gamma (PPARγ) in glucose metabolism and cell proliferation.[38] Particularly, upregulation of platelet isoform of phosphofructokinase (PFKP) by TGFβ1 in HPASMCs promotes vascular SMC glycolysis in human PAH.[38] In addition, glycolysis is significantly upregulated in the PAH lung.[39] Transpulmonary gradients of azelate (nonanedioate) showed a step-down in PAH and a step-up in controls (Fig. 4G). Therefore, we speculate that lower levels of azelate
(nonanedioate) in the pulmonary vasculature/parenchyma/interstitium are associated with a healthy, non-hypertensive phenotype; if true, this would suggest that the observed transpulmonary step-down of plasma azelate (nonanedioate) in PAH is due to its increased uptake into the lung (causing higher levels in the pulmonary vasculature (cells constituting the vascular wall) and the non-vascular lung parenchyma/interstitium).

The **phosphatidylcholines** PC(FA20:5) and PC(18:0/20:5) decreased in PAH, but increased in controls, transpulmonary circulation (AAO vs. PA; Fig. 3B). Phosphatidylcholine is the principal phospholipid known as a major source for the production of arachidonic acid (AA).[40] AA is a fatty acid (20:4), which is normally acetylated in membrane phospholipids.[41] AA is liberated primarily by a cytosolic phospholipase A2 and, via actions of cyclo-oxygenase (COX) and prostacyclin synthase, is involved in production of prostacyclin (PGI2) [41] that inhibits platelet activation and is also a very effective vasodilator. Importantly, prostacyclin is a powerful cardioprotective hormone released by the endothelial cells, maintains equilibrium with other vasoactive hormones; violation of this equilibrium can result in CVDs such as PAH.[41]

**Differential same-site metabolite concentration levels in pediatric pulmonary arterial hypertension**

In the same-site (between-group) comparisons (Fig. 5), glycocholenate sulfate was upregulated in the SVC in PAH plasma. Glycocholenate sulfate is known to be associated with atrial fibrillation in serum of African-Americans.[42] N6-acetyllysine (downregulated in the PA in PAH plasma) is associated with CVD mortality as discussed above.
Metabolite gradients correlate with hemodynamic and echocardiographic variables in PAH

The pediatric values of echocardiography/hemodynamic variables (partly extrapolated from adults) that define a high risk PAH patient are: TAPSE<10 mm Hg (for children older than one year), mPAP/mAAO>0.75, PVRi>15 WU·m⁻², among other criteria[2] Importantly, the alterations of novel circulating metabolite biomarkers for PAH identified in our study correlated with several of prognostic invasive hemodynamic and echocardiographic variables that are essential to diagnosis, prognosis, and outcome in PAH (Tables 2, S4, S5, and S6), as reported in the new pediatric PAH risk score.[17]
Limitations of the study

We followed strict enrollment criteria for pediatric patients undergoing invasive catheterization (see methods). As a result, we have relatively small sample sizes. Furthermore, to increase robustness of our data, we excluded measurements with a missing value in at least one of the three catheterization sites (due to very low concentrations or technical issues). Therefore, we could only unravel the metabolites with large enough concentration gradient difference (Fig. 2 - 4) or concentration difference (e.g., PAH SVC vs. Con SVC, Fig. 5) (i.e., sufficient effect size) to still produce significantly low p-values. However, because the EDTA blood samples were taken from all three catheterization sites within one minute, the design of our gradient analyses allowed us to use each patient as their own control, thereby eliminating between-patient variability and increasing the statistical power. Additionally, healthy pediatric controls are not available for a cardiac catheterization studies for ethical reasons; therefore, we used well-matched, non-PAH patients with mild to moderate LVOTO and indication for cardiac catheterization as controls. As mentioned earlier, based on our metabolite level measurements alone, it is not possible to determine which mechanism (differential release or differential uptake) is driving any step-up or a step-down in the metabolite concentration gradients. Due to the low sample size, we could not estimate the semiquantitative association of the metabolite levels with severity of PAH (WHO functional classes), however, WHO functional class and 6 minute walk distances are difficult to determine in smaller children and thus of limited value in this setting. Further preclinical and prospective clinical studies are needed to explore the biological role and clinical importance of the identified metabolites in the systemic and pulmonary circulation, and in cardiovascular cells (EC, SMC, cardiomyocytes, fibroblasts, and immune cells).
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Table S1 Demographics, diagnosis, and medication of IPAH patients and controls (Metabolon)

| ID | Age category | Weight (kg) | BSA (m²) | WHO Class | Diagnosis | Medication |
|----|--------------|-------------|----------|-----------|-----------|------------|
| 1C | Toddler      | 11.6        | 0.51     | 1         | Moderate AS, moderate AR | VIT D3, IRO |
| 2C | Infant       | 6.5         | 0.33     | 1         | Mediastinal teratoma, no chemotherapy, AAO stenosis | - |
| 3C | Adolescent   | 38.0        | 1.31     | 1         | Severe vAS, mild AR | ASS |
| 4C | Adolescent   | 56.0        | 1.75     | 1         | Moderate vAS, moderate AR | LIS, MET, FSA |
| 5C | Child        | 29.0        | 1.05     | 1         | Moderate vAS, moderate AR | ASS |
| 6C | Infant       | 3.8         | 0.32     | 1         | Severe sAS, AO-Arch-Hypoplasia, imbalanced AV canal, L-SVC, no shunt | PPO, SPI, CLG |
| 7C | Toddler      | 12.7        | 0.53     | 1         | Moderate vAS, trivial AR | VIT C |
| 8C | Child        | 32          | 1.05     | 1         | Portal vein stenosis s/p liver transplantation | TAC, VIT D3, MG, ASS, PRED, IRO |

BSA denotes body surface area; IPAH, idiopathic PAH (Nizza PH category 1.1); AAO, ascending aorta; AGS, adrenogenital syndrome; CLD, chronic lung disease; IVS, intact ventricular septum; L-SVC, persistent left superior vena cava; PDA, patent ductus arteriosus; subAS, subvalvular aortic stenosis; AR, aortic regurgitation; vAS, valvular aortic stenosis; PFO, patent foramen ovale; IVS, intact ventricular septum; AVSD, atrioventricular septal disease; s/p, status post; WHO, World Health Organization; **Medication:** AML, amlodipine; ASA, acetylsalicylic acid; AZI, azythromycin (P.O.); BOS, bosentan (P.O.); DIG, digoxin (P.O.); CHH, chloral hydrate; CLG, clopidogrel (P.O.); FUR, furosemide (Lasix) (P.O.); FSA, fluticasone/salmeterol; HYC, hydrocortison; IBU, ibuprofen (P.O.); IRO, iron (P.O.); ITB, Ipratropium bromide (P.O.); KA, potassium (Rekawan); KI, potassium iodide (P.O.); LAC, lactulose (P.O.); MAC, macitentan (P.O.); MAZ, metamizol (P.O.); MEL, melatonin (P.O.); MG, magnesium (P.O.); NaCl, sodium chloride (P.O.); O₂, oxygen by nasal canula; PC, potassium citrate (P.O.); PEN, pentoxifylline (P.O.); PHE, phenobarbital (P.O.); PPI, proton pump inhibitor (P.O.); PPO, propranolol (P.O.); PRED, prednisone (P.O.); RAP, ramipril (P.O.); RIF, rifaximin (P.O.); RIO, roicuguat (P.O.); SAB, salbutamol (P.O.); SIL, sildenafil (P.O.); SPI, spironolactone (P.O.); UDC, ursodiol; VIT C, D-Fluoretten (P.O.); VIT D3, Vigantolette (P.O.); VIT K, Knoakion (P.O.); ASA, acetylsalicylic acid; CLG, clopidogrel (P.O.); LIS, lisinopril (P.O.); MET = metoprolol (P.O.); MG, magnesium (P.O.); SPI, spironolactone (P.O.); PPO, propranolol (P.O.); TAC, Tacrolimus (P.O.). The **age categories** are defined in years as follows: (0-1): Infant, (1-3): Toddler, (3-5): Pre-school, (5-10): Child, and (10-18): Adolescent.
Table S2. Characteristics of control subjects and PAH patients studied (Lipidyzer)

|                       | Control (non-PAH) (N = 9) | PAH (N = 8) | P-value       |
|-----------------------|---------------------------|-------------|---------------|
| **Demographics**      |                           |             |               |
| Age, years (mean, range) | 9.1 (0.7 -17)             | 7.2 (3 - 18) | n.s.(0.4128)  |
| Male sex, n (%)       | 7 (78)                    | 3 (38)      |               |
| Height (m)            | 122.1 ± 14.3              | 131.4 ± 10.3| n.s.(0.5964)  |
| Weight (kg)           | 26.1 ± 6.3                | 32.0 ± 7.2  | n.s.(0.7000)  |
| BSA (m²)              | 0.93 ± 0.17               | 1.06 ± 0.16 | n.s.(0.6294)  |
| **Disease subtypes (n)** |                         |             |               |
|                       | mild to moderate LVOTO (7), mediasinal teratoma (1), portal vein stenosis (1), IPAH (4), PAH-repaired CHD (2), portopulmonary PH (1), PAH (1) |
| **Key hemodynamics**  |                           |             |               |
| **Cardiac catheterization** |                       |             |               |
| sPAP (mm Hg)          | 21.3 ± 2.1                | 74.0 ± 9.9  | 0.0018        |
| mPAP (mm Hg)          | 14.9 ± 1.5                | 61.1 ± 8.7  | 0.0006        |
| dPAP (mm Hg)          | 9.0 ± 1.4                 | 31.0 ± 8.1  | 0.0388        |
| mPAP/mSAP             | 0.25 ± 0.02               | 0.81 ± 0.12 | 0.0001        |
| mTPG (mm Hg)          | 6.7 ± 1.1                 | 53.8 ± 8.5  | 0.0002        |
| dTPG (mm Hg)          | 1.4 ± 0.6                 | 31.4 ± 8.4  | 0.0113        |
| PVRi (WU·m²)          | 1.67 ± 0.30               | 16.7 ± 3.6  | 0.0003        |
| PVR/SVR               | 0.11 ± 0.02               | 0.82 ± 0.13 | 0.0006        |
| Qpi                   | 4.3 ± 0.31                | 3.72 ± 0.46 | n.s.(0.1672)  |
| Qsi                   | 4.67 ± 0.37               | 3.90 ± 0.54 | n.s.(0.1672)  |
| Op/Qs                 | 0.93 ± 0.04               | 0.98 ± 0.04 | n.s.(0.2359)  |
| mRAP (mm Hg)          | 4.1 ± 0.8                 | 5.8 ± 1.4   | n.s.(0.4835)  |
| **Echo**              |                           |             |               |
| RVAWD (cm)            | 0.30 ± 0.03               | 0.74 ± 0.08 | 0.0002        |
| RVEDD (cm)            | 1.3 ± 0.20                | 2.56 ± 0.24 | 0.0062        |
| TAPSE (cm)            | 1.96 ± 0.08               | 1.61 ± 0.10 | 0.0228        |
| LVEF (%)              | 74.2 ± 2.2                | 66.6 ± 3.7  | n.s.(0.0872)  |

Values are presented as mean ± SEM. A Mann-Whitney U test was applied. P < 0.05 was considered significant. All PAH patients with repaired congenital heart disease (PAH-CHD) had the repair > 12 months prior to cardiac catheterization. Two of the PAH patients had trisomy 21 (all with PAH-repaired CHD; patient ID #7 and #8 in Table S1). BSA denotes body surface area; LVOTO, left ventricular outflow tract obstruction; IPAH, idiopathic PAH; CHD, congenital heart disease; sPAP, systolic pulmonary arterial pressure; mPAP, mean pulmonary arterial pressure; dPAP, diastolic pulmonary arterial pressure; mSAP, mean systemic arterial pressure; mTPG, mean transpulmonary pressure gradient; dTPG, diastolic transpulmonary pressure gradient; PVRi, pulmonary vascular resistance index; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance; Qpi, pulmonary flow index; Qsi, systemic flow index; mRAP, mean right atrial pressure; RVAWD, right ventricular anterior wall diameter; RVEDD, right ventricular end-diastolic diameter; TAPSE, tricuspid annular plane systolic excursion; LVEF, left ventricular ejection fraction, n.s., not significant.
Table S3. Individual patient demographics, WHO functional class, diagnosis, and medication (Lipidyzer).

| ID | Age category | Weight (kg) | BSA (m²) | WHO Class | Diagnosis | Medication |
|----|--------------|-------------|----------|-----------|-----------|------------|
| Non-PAH Controls |
| 1C | Toddler      | 11.6        | 0.51     | 1         | Moderate AS, moderate AR | VIT D3, IRO |
| 2C | Infant       | 6.5         | 0.33     | 1         | Medialateral tumor, no chemotherapy, AAO stenosis | - |
| 3C | Toddler      | 38.0        | 1.31     | 1         | Severe vAS, mild AR | ASS |
| 4C | Adolescent   | 56.0        | 1.75     | 1         | Moderate vAS, moderate AR | LIS, MET, FSA |
| 5C | Child        | 29.0        | 1.05     | 1         | Moderate vAS, moderate AR | ASS |
| 7C | Toddler      | 12.7        | 0.53     | 1         | Moderate vAS, trivial AR | VIT C |
| 8C | Child        | 32          | 1.05     | 1         | Portal vein stenosis s/p liver transplantation | TAC, VIT D3, MG, ASS, PRED, IRO |
| 9C | Child        | 23.6        | 0.92     | 1         | Mild vAS, moderate AR | - |
| 10C| Child        | 24.5        | 0.94     | 1         | Moderate AR, mild vAS | - |
| PAH Patients |
| 1 | Child        | 20.0        | 0.78     | 2         | PAH, PDA (IPAH or young Eisenmenger) | SIL, BOS, ILO, SPI, ASS |
| 2 | Pre-school   | 12.0        | 0.55     | 2         | IPAH | SIL, BOS, ASA |
| 3 | Adolescent   | 26.6        | 1.05     | 2         | IPAH | SIL, BOS, ILO, DIG, PRED, PPI |
| 4 | Adolescent   | 67          | 1.75     | 3         | PAH-CHD (repaired AP window) | SIL, BOS, ILO, SPI, IRO, IRO, BCP |
| 5 | Adolescent   | 58.0        | 1.61     | 3         | PAH, Abernethy syndrome, AGS | SIL, ILO, HCY, RAP, VIT D3, VIT K, RET, KI, LAC, RIF |
| 6 | Adolescent   | 36.4        | 1.25     | 3         | Portopulmonary hypertension (portal vein thrombosis) | SIL, BOS, ILO, SPI, ITB, FUR, VIT D3, SAL, PEN, IBU, MET |
| 7 | Child        | 20.3        | 0.83     | 3         | PH, CLD, s/p PDA closure, Filamin A mutation | SIL, BOS, SPI, oxygen |
| 8 | Pre-school   | 16.6        | 0.67     | 3         | Severe IPAH, s/p syncope, AVT ++, excellent treatment response | SIL, MAC, SPI |

BSA denotes body surface area; IPAH, idiopathic PAH (Nizza PH category 1.1); AAO, ascending aorta; AGS, adrenalogenital syndrome; CLD, chronic lung disease; IVS, intact ventricular septum; L-SVC, persistent left superior vena cava; PDA, patent ductus arteriosus; subAS, subvalvular aortic stenosis; AR, aortic regurgitation; vAS, valvular aortic stenosis; PFO, patent foramen ovale; IVS, intact ventricular septum; AVSD, atrioventricular septal disease; s/p, status post; WHO, World Health Organization; Medication: AML, amlodipine; ASA, acetylsalicylic acid; AZI, azithromycin (P.O.); BOS, bosentan (P.O.); DIG, digoxin (P.O.); CHH, chloral hydrate; CLG, clopidogrel (P.O.); FUR, furosemide (Lasix) (P.O.); FSA, fluticasone/salmeterol ; HVC, hydrocortison; IBU, ibuprofen (P.O.); IRO, iron (P.O.); ITB, Ipratropium bromide (P.O.); KA, potassium (Rekawan); KI, potassium iodide (P.O.); LAC, lactulose (P.O.); MAC, macitentan (P.O.); MAZ, metamizol (P.O.); MG, magnesium (P.O.); NaCl, sodium chloride (P.O.); O₂, oxygen by nasal canula; PC, potassium citrate (P.O.); PHE, phenobarbital (P.O.); PPI, proton pump inhibitor (P.O.); PPO, propranolol (P.O.); Pred, prednisone (P.O.); RAP, ramsipril (P.O.); RIF, rifaximin (P.O.); RIO, riociguat (P.O.); SAB, salbutamol (P.O.); SIL, sildenafil (P.O.); SPI, spironolactone (P.O.); UDC, ursodiol; VIT C, D-Fluoretten (P.O.); VIT D3, Vigantollette (P.O.); VIT K, Knoakion (P.O.); ASA, acetylsalicylic acid; CLG, clopidogrel (P.O.); LIS, isosorbid (P.O.); MET = metoprol (P.O.); MG, magnesium (P.O.); SPI, spironolactone (P.O.); PPO, propranolol (P.O.); TAC, Tacrolimus (P.O.). The age categories are defined in years as follows, [0-1): Infant, [1-3): Toddler, [3-5): Pre-school, [5-10): Child, and [10-19): Adolescent.
Table S4. Differential trans-RV metabolite concentration gradients (including selected gradient ratios) and their correlation with invasive hemodynamic / echocardiographic variables

| Metabolite or metabolite ratio | Fold change Control | Fold change PAH | FDR-adjusted p-val | Selected hemodynamic or echocardiographic variables | r       | p-val  |
|-------------------------------|---------------------|-----------------|--------------------|--------------------------------------------------|---------|--------|
| Subclass: Glycerophosphoethanolamines |                     |                 |                    |                                                  |         |        |
| 1-palmitoyl-GPE (16:0)         | 0.70                | 2.31            | 0.0134             | TAPSE, cm                                      | -0.73   | 0.0254 |
|                                |                     |                 |                    | sPAP, mm Hg                                     | 0.55    | 0.0424 |
| 1-palmitoyl-GPE (16:0) / 1-linoleoyl-GPE (18:2) | 0.61                | 2.18            | 0.0092             | TAPSE, cm                                      | -0.73   | 0.0258 |
|                                |                     |                 |                    | sPAP, mm Hg                                     | 0.53    | 0.0489 |
| 1-palmitoyl-GPE (16:0) / margarate (17:0) | 0.55                | 2.02            | 0.0092             | TAPSE, cm                                      | -0.68   | 0.0632 |
|                                |                     |                 |                    | sPAP, mm Hg                                     | 0.54    | 0.0523 |
| 1-palmitoyl-GPE (16:0) / palmitate (16:0) | 0.64                | 2.16            | 0.0099             | TAPSE, cm                                      | -0.67   | 0.0473 |
|                                |                     |                 |                    | sPAP, mm Hg                                     | 0.52    | 0.0577 |
| 1-palmitoyl-GPE (16:0) / stearate (18:0) | 0.64                | 2.10            | 0.0099             | TAPSE, cm                                      | -0.71   | 0.0314 |
|                                |                     |                 |                    | sPAP, mm Hg                                     | 0.53    | 0.0533 |
| 1-palmitoyl-GPE (16:0) / 1-oleoyl-GPE (18:1) | 0.77                | 1.50            | 0.0141             | sPAP, mm Hg                                     | 0.53    | 0.0533 |
| Subclass: Glycerophosphoethanolamines |                     |                 |                    |                                                  |         |        |
| Role: Phospholipid biosynthesis, Glycerophospholipid metabolism, Lipid metabolism |                     |                 |                    |                                                  |         |        |
| 1-oleoyl-GPE (18:1)            | 0.78                | 1.48            | 0.0767             | TAPSE, cm                                      | -0.83   | 0.0104 |
|                                |                     |                 |                    | sPAP                                            | -0.52   | 0.0533 |
| Subclass: Fatty acids and conjugates |                     |                 |                    |                                                  |         |        |
| Role: Lipid transport, Lipid metabolism, Fatty acid metabolism |                     |                 |                    |                                                  |         |        |
| Octadecanedioate               | 0.77                | 1.09            | 0.0157             | TAPSE, cm                                      | -0.72   | 0.0444 |
|                                |                     |                 |                    | RVAWD, cm                                       | 0.52    | 0.0560 |
|                                |                     |                 |                    | Tricuspid valve E/A                             | -0.71   | 0.0226 |
|                                |                     |                 |                    | mPAP, mm Hg                                     | 0.56    | 0.0371 |
|                                |                     |                 |                    | PVRI, WU·m²                                      | 0.54    | 0.0565 |
| Subclass: Fatty acid esters |                     |                 |                    |                                                  |         |        |
| Role: Lipid transport, Lipid metabolism, Fatty acid metabolism |                     |                 |                    |                                                  |         |        |
| Stearoylcarnitine              | 0.74                | 1.21            | 0.0582             | sPAP, mm Hg                                     | 0.52    | 0.0814 |

For gradient analysis, the p-values were generated using Type II Wald chi-square test (FDR<0.15). Abbreviations: FDR, false discovery rate; TAPSE, tricuspid annular plane systolic excursion; sPAP, systolic pulmonary arterial pressure; mPAP, mean right pulmonary arterial pressure; mPAP, mean pulmonary arterial pressure; PVRI, pulmonary vascular resistance index; RVAWD, right ventricular anterior wall diameter; mAAO, mean ascending aorta pressure.
Table S5. Differential transpulmonary metabolite concentration gradients and their correlation with invasive hemodynamic / echocardiographic variables

| Metabolite or metabolite ratio | Fold change Control | Fold change PAH | FDR-adjusted p-val | Selected hemodynamic or echocardiographic variables | r      | p-val  |
|-------------------------------|---------------------|----------------|-------------------|-----------------------------------------------|--------|-------|
| **Subclass: Amino acids, peptides, and analogues** |                     |                |                   |                                               |        |       |
| **Role: Lysine metabolism**   |                     |                |                   |                                               |        |       |
| N6-acetylysine                | 0.71                | 1.53           | 0.0476            | TAPSE, cm                                     | -0.94  | 0.0174|
|                               |                     |                |                   | RVSP, mmHg                                     | 0.63   | 0.0657|
|                               |                     |                |                   | RVAWD, cm                                      | 0.74   | 0.0090|
|                               |                     |                |                   | mPAP, mm Hg                                    | 0.78   | 0.0029|
|                               |                     |                |                   | mTPG, mm Hg                                    | 0.74   | 0.0056|
|                               |                     |                |                   | dTPG, mm Hg                                    | 0.78   | 0.0027|
|                               |                     |                |                   | mPAP/mAAO                                      | 0.84   | 0.0006|
|                               |                     |                |                   | PVRI, WU·m²                                     | 0.73   | 0.0067|
| **Subclass: Glycerophosphocholines** |                 |                |                   |                                               |        |       |
| **Role: Phospholipid metabolism, Lipid transport, Lipid metabolism, Fatty acid metabolism** |                 |                |                   |                                               |        |       |
| 2-palmitoyl-GPC (16:0)        | 1.22                | 0.83           | 0.0184            | TAPSE, cm                                     | 0.86   | 0.0058|
|                               |                     |                |                   | RVAWD, cm                                      | -0.53  | 0.0523|
|                               |                     |                |                   | RVEDD, cm                                      | -0.53  | 0.0633|
|                               |                     |                |                   | mRAP, mm Hg                                    | -0.54  | 0.0675|
|                               |                     |                |                   | mPAP, mm Hg                                    | -0.47  | 0.0881|
|                               |                     |                |                   | mPAP/mAAO                                      | -0.55  | 0.0383|
|                               |                     |                |                   | PVRI, WU·m²                                     | -0.51  | 0.0730|
|                               |                     |                |                   | PVRI/SVR                                       | -0.65  | 0.0296|
| **Subclass: Hybrid peptides** |                     |                |                   |                                               |        |       |
| **Role: Free radical scavenger, Lipid peroxidation suppression** |                 |                |                   |                                               |        |       |
| N-acetylcarnosine             | 1.05                | 0.72           | 0.0638            | PAWP, mm Hg                                    | 0.75   | 0.0327|
| **Subclass: Fatty acids and conjugates** |                 |                |                   |                                               |        |       |
| **Role: Lipid transport, Lipid metabolism, Fatty acid metabolism** |                 |                |                   |                                               |        |       |
| Azelate (nonanedioate)        | 1.22                | 0.42           | 0.0006            | mPAP, mm Hg                                    | -0.68  | 0.0611|
|                               |                     |                |                   | dTPG, mm Hg                                    | -0.75  | 0.0845|

For gradient analysis, the p-values were generated using Type II Wald chi-square test (FDR<0.15). Abbreviations: FDR, false discovery rate; mPAP, mean pulmonary arterial pressure; mTPG, mean transpulmonary pressure gradient; dTPG, diastolic transpulmonary pressure gradient; mAAO, mean ascending aorta pressure; PVRI, pulmonary vascular resistance index; RVAWD, right ventricular anterior wall diameter; TAPSE, tricuspid annular plane systolic excursion; RVSP, right ventricular systolic pressure; mRAP, mean right atrial pressure; SVR, systemic vascular resistance; RVEDD, right ventricular end-diastolic diameter; PAWP, pulmonary arterial wedge pressure.
Table S6. Metabolites with differential concentrations in a single catheterization site (SVC, PA, AAO) correlated with hemodynamics

| Metabolite or metabolite ratio | Catheterization site | Fold change (PAH vs control) | FDR-adjusted p-val | Selected hemodynamic or echocardiographic variables | rho | p-val |
|--------------------------------|----------------------|-----------------------------|--------------------|---------------------------------------------------|-----|-------|
| **Subclass: Fatty acids and conjugates** |                      |                             |                    |                                                   |     |       |
| **Role: Lipid transport, Lipid metabolism, Fatty acid metabolism** |                    |                             |                    |                                                   |     |       |
| Heptanoate (7:0) | SVC | 1.91 | 0.0855 | RVAWD, cm | 0.73 | 0.0091 |
|                  |   |     |        | RVSP, mm Hg | 0.65 | 0.0066 |
|                  |   |     |        | mPAP, mm Hg | 0.86 | 0.0135 |
|                  |   |     |        | mPAP/mAAO | 0.61 | 0.0303 |
|                  |   |     |        | PVRL, WU-m² | 0.63 | 0.0324 |
|                  |   |     |        | PVRSVR | 0.72 | 0.0017 |
| Caproate (6:0) | SVC | 1.54 | 0.1499 | mPAP, mm Hg | 0.51 | 0.0761 |
|                  |   |     |        | PVRL, WU-m² | 0.51 | 0.0936 |
| **Subclass: Bile acids, alcohols and derivatives** |      |   | | | |
| **Role: Secondary bile acid metabolism** |                      |                             |                    |                                                   |     |       |
| Glycocholenate sulfate | SVC | 1.94 | 0.0883 | RVAWD, cm | 0.49 | 0.0750 |
|                  |   |     |        | mPAP, mm Hg | 0.49 | 0.0631 |
|                  |   |     |        | mTPG, mm Hg | 0.49 | 0.0764 |
|                  |   |     |        | mPAP/mAAO | 0.58 | 0.0253 |
| **Subclass: Glycerophosphoinositols** |                      |                             |                    |                                                   |     |       |
| **Role: Phospholipid metabolism, Lipid transport, Lipid metabolism, Fatty acid metabolism** |                      |                             |                    |                                                   |     |       |
| 1-stearoyl-GPI (18:0) | PA | 3.85 | 0.0719 | TAPSE, cm | -0.64 | 0.0831 |
|                  |   |     |        | mPAP, mm Hg | 0.58 | 0.0294 |
|                  |   |     |        | mTPG, mm Hg | 0.55 | 0.0500 |
|                  |   |     |        | mPAP/mAAO | 0.50 | 0.0721 |
|                  |   |     |        | PVRL, WU-m² | 0.57 | 0.0473 |
| 1-stearoyl-GPI (18:0) / dihomo-linolenate (20:3n3 or n6) | PA | 3.46 | 0.0012 | RVAWD, cm | 0.50 | 0.0602 |
|                  |   |     |        | mPAP, mm Hg | 0.66 | 0.0056 |
|                  |   |     |        | mTPG, mm Hg | 0.67 | 0.0063 |
|                  |   |     |        | dTPG, mm Hg | 0.59 | 0.0268 |
|                  |   |     |        | mPAP/mAAO | 0.66 | 0.0062 |
|                  |   |     |        | PVRL, WU-m² | 0.72 | 0.0033 |
| 1-stearoyl-GPI (18:0) / dihomo-linoleate (20:2n6) | PA | 3.51 | 0.0052 | mPAP, mm Hg | 0.50 | 0.0609 |
|                  |   |     |        | PAWP, mm Hg | 0.55 | 0.0323 |
|                  |   |     |        | mTPG, mm Hg | 0.53 | 0.0236 |
|                  |   |     |        | PVRL, WU-m² | 0.60 | 0.0195 |
|                  |   |     |        | PVRSVR | 0.54 | 0.0611 |
| 1-stearoyl-GPI (18:0) / arachidonate (20:4n6) | PA | 2.94 | 0.0072 | TAPSE, cm | -0.77 | 0.0152 |
|                  |   |     |        | mPAP/mAAO | 0.54 | 0.0338 |
|                  |   |     |        | PVRL, WU-m² | 0.53 | 0.0454 |
|                  |   |     |        | PVRSVR | 0.54 | 0.0581 |
| 1-stearoyl-GPI (18:0) / 1-arachidonoyl-GPI (20:4) | PA | 2.30 | 0.0098 | TAPSE, cm | -0.87 | 0.0023 |
|                  |   |     |        | mPAP/mAAO | 0.51 | 0.0484 |
|                  |   |     |        | PVRL, WU-m² | 0.48 | 0.0759 |
| 1-stearoyl-GPI (18:0) / 10-heptadecenoate (17:1n7) | PA | 4.52 | 0.0102 | TAPSE, cm | -0.77 | 0.0265 |
|                  |   |     |        | PVRL, WU-m² | 0.52 | 0.0706 |
|                  |   |     |        | PVRSVR | 0.59 | 0.0458 |
| **Subclass: Amino acids, peptides, and analogues** |                      |                             |                    |                                                   |     |       |
| **Role: Lysine metabolism** |                      |                             |                    |                                                   |     |       |
| N6-acetyllysine | PA | 0.49 | 0.0944 | RVSP, mm Hg | -0.70 | 0.0311 |
|                  |   |     |        | RVAWD, cm | -0.79 | 0.0023 |
|                  |   |     |        | RVEDD, cm | -0.65 | 0.0208 |
|                  |   |     |        | mPAP, mm Hg | -0.68 | 0.0072 |
|                  |   |     |        | mTPG, mm Hg | -0.73 | 0.0045 |
|                  |   |     |        | dTPG, mm Hg | -0.60 | 0.0387 |
|                  |   |     |        | mPAP/mAAO | -0.61 | 0.0225 |
|                  |   |     |        | PVRL, WU-m² | -0.77 | 0.0033 |
|                  |   |     |        | PVRSVR | -0.72 | 0.0150 |

For between-group comparisons, Mann-Whitney U test was used, FDR<0.15. Abbreviations: FDR, false discovery rate; mPAP, mean pulmonary arterial pressure; mAAO, mean ascending aorta pressure; PVRL, pulmonary vascular resistance index; SVR, systemic vascular resistance; RVAWD, right ventricular anterior wall diameter; RVSP, right ventricular systolic pressure; mTPG, mean transpulmonary pressure gradient; TAPSE, tricuspid annular plane systolic excursion; PAWP, pulmonary arterial wedge pressure.
Table S7. Trans-RV fold changes (FC), p-values, and FDR-adjusted p-values (q-values) of the metabolites in the Discussion of the main manuscript.

| Metabolite                  | FC PAH | FC Con | p-value       | q-value       |
|-----------------------------|--------|--------|---------------|---------------|
| 1-palmitoyl-GPE (16:0)      | 2.3103 | 0.7031 | 0.000536916   | 0.013276246   |
| 1-oleoyl-GPE (18:0)         | 1.4797 | 0.7813 | 0.005723868   | 0.043001007   |
| octadecanedioate             | 1.09   | 0.7679 | 0.002980109   | 0.043001007   |
| dodecanedioate               | 1.07   | 0.7227 | 0.04943325725 | 0.3245496113  |
| tetradecanedioate            | 1.2715 | 0.84   | 0.2517646426  | 0.6765543234  |
| TAG54:7-FA22:6               | 1.4449 | 0.7746 | 0.005475415   | 0.104955657   |
| DAG(FA22:6)                  | 1.4728 | 0.9476 | 0.001995479   | 0.043001007   |
| arachidate (20:0)            | 1.3382 | 0.9749 | 0.03300509845 | 0.2499439809  |