



# Microarray analysis in pulmonary hypertension

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**ABSTRACT** Microarrays are a powerful and effective tool that allows the detection of genome-wide gene expression differences between controls and disease conditions. They have been broadly applied to investigate the pathobiology of diverse forms of pulmonary hypertension, namely group 1, including patients with idiopathic pulmonary arterial hypertension, and group 3, including pulmonary hypertension associated with chronic lung diseases such as chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. To date, numerous human microarray studies have been conducted to analyse global (lung homogenate samples), compartment-specific (laser capture microdissection), cell type-specific (isolated primary cells) and circulating cell (peripheral blood) expression profiles. Combined, they provide important information on development, progression and the end-stage disease. In the future, system biology approaches, expression of noncoding RNAs that regulate coding RNAs, and direct comparison between animal models and human disease might be of importance.



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**Comprehensive overview of compartment-specific microarray studies of material from pulmonary hypertension patients** <http://ow.ly/YEFO2>

Received: Dec 03 2015 | Accepted after revision: Feb 15 2016 | First published online: April 13 2016

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## Introduction

Pulmonary hypertension (PH) includes a large spectrum of diseases with elevated pulmonary pressures  $\geq 25$  mmHg. PH is characterised by pulmonary vascular remodelling and associated with high resistance to blood flow in the lung, which ultimately leads to right heart failure and death [1]. PH can manifest as: pulmonary arterial hypertension (PAH) (group 1); PH due to left heart disease (group 2), chronic lung disease (CLD) and/or hypoxia (group 3); chronic thromboembolic PH (group 4); and PH with unclear multifactorial mechanisms (group 5) [1]. PH is a frequent (up to 60% prevalence) and severe complication of CLD [2]. The occurrence of PH is an indicator of disease progression and predicts patients' outcome [2–4]. The main pathophysiological hallmark of PAH and PH with CLD is pulmonary vascular remodelling of small pulmonary arteries. This includes, most importantly, intimal hyperplasia, medial thickening due to pulmonary artery smooth muscle cell (PASMC) proliferation and, to some extent, adventitial remodelling [5, 6]. Another feature of PH is intra- and perivascular inflammation leading to activation of growth factor signalling pathways, and proliferation of PASMCs, which further potentiates arterial remodelling [7]. Circulating cells and their mediators have also been postulated to be involved in disease progression as they are capable of promoting recruitment, retention and differentiation of circulating monocytic cell populations that contribute to vascular remodelling [8, 9]. Although the understanding of PH pathobiology has increased substantially over recent years there is still a pressing need to fully comprehend how underlying mechanisms drive vascular remodelling.

## RNA expression studies

Gene expression studies, such as microarrays and RNA sequencing, provide accessible and fast screening technologies to detect genes, groups of co-regulated genes or pathways that are involved in remodelling processes. They allow for a broad and unbiased look at the differential whole-genome gene expression patterns in PH. To date, RNA expression studies have been employed to 1) identify genes and pathways that have previously not been associated with PH pathogenesis [10], 2) detect new potential biomarkers [11], 3) identify individuals at risk for developing PH [12], and 4) determine the impact of medication on disease progression [13].

In addition to identifying coding RNAs (mRNA), the expression of noncoding RNAs such as microRNAs (miRNAs) can also be analysed. Unlike coding mRNAs, noncoding RNAs are not translated to proteins [14, 15] but can regulate expression of mRNAs at the transcriptional and post-transcriptional level [14]. Noncoding RNAs involved in epigenetic processes can be divided into two main groups: short noncoding (e.g. miRNAs <30 nt) and long noncoding RNAs (>200 nt) [16]. While short noncoding RNAs have attracted some attention in recent studies [11, 17], the information on expression, function and role of long noncoding RNAs in PH is still limited.

## Microarray technology and data analyses

Microarray technology has been used for more than two decades, and is today well established and highly standardised on the level of instrumentation and biochemistry [18, 19]. Additionally, the majority of research uses microarrays to study gene expression. For these reasons, this review focuses on studies utilising microarray technology only.

Microarrays are tools to measure large numbers of different sequences in a complex mixture of nucleic acids. The RNA samples are amplified, labelled and hybridised to an array of spotted oligonucleotides. Image analysis identifies the spots, quantifies the signals and constructs data tables including the spot annotations that can be further processed and analysed. The data processing can include background subtraction and normalisation to adjust intensity profiles of different arrays. To identify “candidate genes” that are likely to be differentially expressed between groups or conditions, genes can be ranked by their average (logarithmically transformed) fold change or by a (possibly moderated) t-statistic, and the top-ranking genes can be identified. It is also common practice to create lists of candidate genes with a given false-discovery rate (the expected proportion of false positives among the actually rejected null hypotheses) [20]. Afterwards, genes can be analysed for co-expression patterns by clustering, multidimensional scaling and principal component analysis to identify gene sets that may be involved in similar physiological processes. Externally defined gene sets, for instance, genes belonging to a particular signalling pathway, can be analysed for statistical enrichment among the top-ranked genes (gene-set enrichment analysis) or statistical over-representation of candidate genes (over-representation analysis) [21].

## Challenges of microarray studies

The difficulty in studying genes involved in pulmonary vascular remodelling may be confounded by several factors that can influence the results, such as the method of specimen acquisition and storage, RNA quality [22], pre-amplification [23], labelling protocols [24], microarray platform [25], and data analyses applied [26–29]. Additionally, the interpretation of data is prone to bias. The selection criterion is

subjective, and a good choice depends on the context, the design and the aim of the experiment. Numerous genes are often identified as differentially regulated. Picking genes that are indeed relevant to disease pathogenesis is challenging. Mostly, the strongest differentially regulated genes are selected for further analyses. Whether these are also the genes which are biologically relevant is unpredictable. Less regulated but pathophysiologically important genes might easily be overlooked. Therefore, methods such as clustering, gene set or network analyses use the information from many genes in parallel and are thus much more robust against chance variation of individual genes. The integration of additional, external knowledge about the biology of the genes can generate hypotheses about relevant biological processes that are mirrored by the changes of the expression profiles.

The relevance of differences in gene expression detected by microarray analyses can only be estimated when combining microarray results with validation on protein level by western blotting or flow cytometry. The confirmation of findings on protein level is crucial as deregulated genes do not automatically lead to changes on protein level due to control of the proteome fingerprint by miRNAs and noncoding RNAs.

### **Selection criteria**

This review attempts to summarise the current and relevant literature focusing on microarray based high-throughput transcriptome analyses of idiopathic PAH (IPAH) (as a prototype disease), familial pulmonary arterial hypertension (FPAH) and PH associated with CLD such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). Human microarray studies on PH groups 2, 4 and 5 are limited [30], and are not included in this review. As microarray data can be derived from the analyses of different compartments of the lung, we have summarised the current status of data collected by microarray analyses of human lung homogenate samples, laser capture microdissected pulmonary arteries, and isolated or circulating cells (table 1).

The selection criteria for studies included in this review were article type (research articles published until November 2015), species (human), technology (microarray or gene array) and disease (group 1 and 3 PH or PAH). A short synopsis of rodent PH model microarray studies is provided at the end of this review.

### **Microarray studies on human lung tissue**

Explanted lungs (representing the end-stage disease) or biopsy material (representing a point in time during disease progression but usually not clinically indicated and, therefore, rarely available) provide a unique opportunity to identify molecular mechanisms and potentially new therapeutic targets in the diseased lung. Although the access to human explants is rather limited, it is an even greater challenge to obtain the necessary healthy donor material for comparison. The source of control tissue can originate from downsizing of transplanted lungs, nontransplanted lungs or resection of tumours. This can bias the results of microarray analyses. Similarly, other confounding factors such as the severity of the disease, comorbidities, medication and the age/sex of the cohort might influence study outcome.

The easiest but least refined approach when analysing gene expression in PH is the analysis of samples from total lung homogenate. As intrapulmonary arteries represent only a minor portion of lung tissue (<10%), the expression profile of this compartment may be largely masked or even lost when analysing lung homogenates [50]. Inevitably, the use of whole tissue results in the average of the various expression profiles coming from diverse cell types. Additionally, lung sections may contain vessels of different sizes and may have differing cell composition, which makes it difficult to interpret the results with regard to relevant biological processes. Furthermore, severe parenchyma remodelling in CLD may disguise relevant findings. Nevertheless, utilisation of lung homogenate can deliver insights on gene expression that, by further immunohistochemical validation, can lead to discovery of new potential targets involved in vascular remodelling. In order to specifically analyse gene expression in the pulmonary vasculature, several studies have applied laser capture microdissection (LCM) to isolate small pulmonary arteries [51, 52]. Another approach to overcome tissue heterogeneity is to analyse isolated primary human cells such as pulmonary artery endothelial cells, PASMCs or fibroblasts. However, here, several aspects have to be taken into consideration as changes in gene expression can be affected by isolation and cell culture conditions. The impact of passaging cultured cells on gene expression is well known [53–55]. Growth factors in the culture medium can alter paracrine and hormone signalling and cell growth, and thereby can influence gene expression [56, 57]. Finally, analysis of circulating cells can provide a useful tool to detect novel biomarkers of disease progression or monitor personalised medication. Due to their clinical accessibility, circulating blood cells represent the most convenient cell source for the assessment of alteration in gene expression in PH.

### **Lung homogenate samples**

Using human lung homogenate samples of IPAH and FPAH, GERACI *et al.* [31] revealed decreased expression of genes encoding kinases and phosphatases, and upregulation of oncogenes and genes coding

TABLE 1 Details of microarray studies published on lung homogenate (LH), laser capture microdissection (LCM), and isolated or circulating cells since 2001 with a focus on pulmonary arterial hypertension (PAH) or pulmonary hypertension (PH) associated with chronic lung diseases, displayed in chronological order

	Patient cohort	Mean age years	Mean mPAP mmHg	Biological material	Target molecule	Platform	Prominent genes	Prominent pathways/processes	First author [ref.]	GEO link
LH samples	6 controls (with the primary diagnosis of cervical carcinoma, adenocarcinoma of the lung, unknown primary diagnosis, head trauma, synovial cell carcinoma and lung carcinoma)	NA		LH, total RNA	mRNA	Affymetrix <sup>®</sup>	<i>TGFBR3, BMP2, MAPKK5, RACK1, APOC3, LAMR1/RPSA</i>	Protein synthesis and degradation Endothelial cell biology Cell growth and apoptosis	GERADI [31]	NA
	6 PPH subjects (2 FPPH)	37	59							
	13 normal controls	60		LH, total RNA	mRNA	Agilent <sup>®</sup>	<i>BMPR1A, BMPR2, PTGS2, VEGFA, ADAMTS9, CHL1, CYBB/NOX2, E2F1, ESR1, F2RL3, MYBL1, NCOA2, P2RY1, PF4, PLN, PPP2CA, TMOD3</i>	Regulation of actin-based motility by Rho cAMP-mediated signalling Protein ubiquitination pathway	RAJUMAR [32]	GSE15197
	18 PAH subjects	44	55							
	8 IPF subjects with secondary PH	60	45							
	9 normal controls (normal lung tissue specimens were obtained from organ donors whose lungs were not used for lung transplantation)	53		LH, total RNA	mRNA	Illumina <sup>®</sup>	<i>CXCL10, FCN3, IGFBP7, SCGB1A1</i>	Fibrosis IGF signalling caveolin-mediated endocytosis antigen presentation chemokine activity IL-17 signalling	Hsu [33]	GSE48149
	9 SSc-PF subjects	49	20							
LCM	9 SSc-PAH subjects	52	48							
	10 IPF subjects	62	23							
	8 IPAH subjects	35	60							
	17 PH-IPF subjects	58	48	LH, total RNA	mRNA	Affymetrix	<i>SPPI, MMP1, MMP7, MMP13, EDNRB, PTX3, S100A8</i>	PH phenotype: cell proliferation, fibroblast migration noPH phenotype: proinflammatory genes	MURA [12]	GSE24988
	45 intermediate PH-IPF subjects	59	29							
	22 IPF subjects	61	17							
	7 IPAH subjects	43	53							
Isolated cells	8 controls	47		LH, total RNA	mRNA	Affymetrix	<i>CYP7B1</i>	Bile acids metabolites	ZHAO [34]	GSE53408
	8 PAH subjects	40	NA							
	6 donors (nontransplanted donor lung tissue showing no evidence of vascular pathology)	47		PAAs <500 µm, total RNA	mRNA	Agilent	<i>DAAM1, RAC1, RHOA, ROCK, DSV, WNT11</i>	Extracellular matrix and cytoskeleton Immune reaction WNT signaling pathway	LAUMANN [35]	GSE10704
	6 IPAH subjects	28	102							
	7 donors (4 unused donor lung shavings and 3 normal lung sections obtained during resection of pulmonary carcinoid tumours)	45		PAAs 100–450 µm, total RNA	mRNA	Affymetrix	<i>STAT1, SMOC2, SFRP2</i>	Amino acid metabolism AMP kinase signalling Ciliary neurotrophic factor signalling Complement system Cellular growth/proliferation	PATEL [36]	NA
	8 PH-IPF subjects	59	29							
	8 non-PH-IPF subjects	63	12							
Isolated cells	8 donors (downsized nontumorous nontransplanted donor lungs)	44		PAAs <300µm, total RNA	mRNA	Agilent	<i>COL3A1, TNC, COL6A3, THBS2, VWF</i>	Retinol metabolism ECM receptor interaction	HOFFMANN [6]	NA
	8 PH-IPF subjects	62	55							
	8 PH-COPD subjects	57	53							
	2 normal controls	NA		PASMCs treated with BMP-2, 200 nM for 24 h; passages 5–6	mRNA	Affymetrix	<i>CAV2, MYCBP, FOS, ANXA5, CYC1, GATA2, TGFB1, TGFB2A, GADD34, HTR2B, F2R, ITPR1</i>	Cell growth and contraction Apoptosis	FANTOZZI [37]	GSE2559
	2 IPAH subjects	44	52							

Continued

TABLE 1 Continued

	Patient cohort	Mean age years	Mean mPAP mmHg	Biological material	Target molecule	Platform	Prominent genes	Prominent pathways/processes	First author [ref.]	GE0 link
	3 normal controls	NA		HMVEC-L and HMVEC-C treated with 3, 24 or 48 h hypoxia; passages 6–7	mRNA	Affymetrix	<i>GREM1, LIMCH1, SLM2, SPON1, ENPP2, PRKY, MPEG1, TM2D1, CXCR7, MYO1B, BDNF</i>		COSTELLO [38]	GSE11341
	9 normal controls	53		Fibroblasts, passage 3; total RNA	mRNA	Illumina	<i>PODN, LOX, FHL2</i>	Fibrosis	Hsu [33]	GSE48149
	9 SSc-PF subjects	49	20					IGF signalling		
	9 SSc-PAH subjects	52	48					Caveolin-mediated endocytosis		
	10 IPF subjects	62	23					Antigen presentation		
	6 IPAH subjects	35	60					Chemokine activity		
								Interleukin-17 signalling		
	3 normal controls	NA		Primary HPASMCs treated with 24 and 48 h hypoxia; passages 6 and 8; miRNA	miRNA	Exiqon <sup>®</sup>	<i>miR-210</i>		Gou [39]	NA
	3 controls (no history of pulmonary or cardiac disease or symptoms)	NA		HPASMCs up to passage 6, total RNA	mRNA	Affymetrix	<i>BKB2R, PLK1, PLK4, ANLN, ANGPT1, DAPK1, SOD3</i>	Cellular growth/proliferation/maintenance/movement Cell cycle/death/survival	Yu [40]	NA
	3 IPAH subjects	NA	NA							
	3 HPAH subjects	NA	NA							
Circulating cells	6 normal controls	39		PBMCs, total RNA	mRNA	Affymetrix	<i>ECGF1, ADM, HVEM</i>	Inflammatory response	BULL [41]	GSE703
	7 IPAH subjects	43	60					Response to stress		
	8 sPAH subjects	57	50					Cytochrome C oxidase		
								Lysosome		
								Intracellular signalling cascade		
	5 healthy Caucasian controls	47		PBMCs, total RNA	mRNA	Affymetrix	<i>DEFA1, DEFA3, S100P, PROK2</i>	Inflammation	ULRICH [42]	NA
	5 severe IPAH subjects	46	51					Immune responses		
	5 healthy controls	34		PBMCs, total RNA	mRNA	Affymetrix	<i>MMP9, VEGF, EREG, IL8</i>	Angiogenesis	GRIGORYEV [43]	NA
	9 IPAH subjects	49	50					Chemotaxis		
	10 PAH-SSc	61	51					Inflammation		
	3 unaffected <i>BMPR2</i> mutation carriers	73	NA	Cultured lymphocytes, total RNA	mRNA	Affymetrix	<i>CYP1B1</i>	Stress response	WEST [44]	GSE10767
	4 affected <i>BMPR2</i> mutation carriers	22	NA					Actin organisation		
								Proliferation		
								Ras-related G-proteins		
								Calcium balance		
	10 healthy controls	NA		PBMCs, total RNA	mRNA	Agilent	<i>ICAM1, IFNGR1, IL1B, IL13RA1, JAK2, AIF1, CCR1, ALAS2, TIMP2</i>	Vascular injury	PENDERGRASS [45]	GSE19617
	21 ISSc subjects without PAH	55	19					Proliferation		
	15 ISSc-PAH subjects	70	47					Inflammatory responses		
	10 SSc	51	NA	PBMCs, total RNA	mRNA	Affymetrix	<i>IL7R</i>	Inflammation	RISBANO [46]	GSE22356
	10 SSc-PAH	62	80					Immunity		
	41 healthy controls	45		PBMCs, total RNA	mRNA	Illumina	<i>ALAS2, ERAF/AHSP</i>	Erythrocyte maturation	CHEADLE [47]	GSE33463
	30 IPAH subjects	52	NA							
	19 SSc subjects	55	NA							
	42 SSc-PAH subjects	60	NA							
	8 SSc-PH-ILD subjects	61	NA							

Continued

TABLE 1 Continued

Patient cohort	Mean age years	Mean mPAP mmHg	Biological material	Target molecule	Platform	Prominent genes	Prominent pathways/processes	First author [ref.]	GEO link
8 healthy controls	40		Plasma, miRNA	miRNA	Geniom <sup>f</sup>	<i>miR-150</i>		RHOES [11]	NA
8 treatment-naïve PAH subjects	40	57							
8 healthy controls	NA	17	Plasma, miRNA	miRNA	NA	<i>miR-451, miR-1246, miR-23b, miR-130a, miR-191</i>		Wei [17]	NA
15 moderate PH subjects	NA	29							
17 severe PH	NA	46							
28 controls	43		PBMCs, total RNA	mRNA	Agilent	<i>TCF7, IL7R</i>	Metabolic process T-cell receptor signalling pathway	CHESNE [48]	GSE38267
13 PAH subjects	41	67							
23 CF subjects	24	NA							
22 healthy controls (free from known cardiovascular disease)	NA		Blood-derived lymphocyte culture, 6 weeks; total RNA	mRNA	Affymetrix	<i>DSG2, RHOQ</i>	Cytoskeletal/Rho GTPase genes Cell adhesion genes Transcription factors Developmental pathways	HEMNES [13]	NA (series pending)
32 idiopathic and heritable VN-PAH subjects	37	60							
8 idiopathic and heritable VR-PAH subjects	27	46							
10 controls	NA		Plasma, miRNA	miRNA	Affymetrix	<i>miR-23a</i>		SARRION [49]	NA
12 IPAH subjects	56	49							

GEO: Gene Omnibus; NA: not available; PPH: primary pulmonary hypertension; FPPH: familial primary pulmonary hypertension; mPAP: mean pulmonary artery pressure; IFP: idiopathic pulmonary fibrosis; SSC: systemic sclerosis; PF: pulmonary fibrosis; IPAH: idiopathic pulmonary arterial hypertension; COPD: chronic obstructive pulmonary disease; HPAH: heritable pulmonary arterial hypertension; sPAH: secondary pulmonary arterial hypertension; *BMPR2*: bone morphogenetic protein receptor II; *ISSC*: limited systemic sclerosis; *ILD*: interstitial lung disease; *CF*: cystic fibrosis; *VN*: vasodilator-nonresponsive; *VR*: vasodilator responsive; *PA*: pulmonary artery; *PASMC*: pulmonary artery smooth muscle cell; *BMP*: bone morphogenetic protein; *HMVEC-L*: human lung microvascular endothelial cell; *HMVEC-C*: human cardiac microvascular endothelial cell; *HPASMC*: human pulmonary smooth muscle cell; *PMBC*: peripheral blood mononuclear cell; *miRNA*: microRNA; *TGFBR3*: transforming growth factor- $\beta$  receptor III; *BMP2*: bone morphogenetic protein 2; *MAPKK5*: mitogen-activated protein kinase kinase 5; *RACK1*: receptor for activated C kinase 1; *APOC3*: apolipoprotein C-III; *LAMR1*: laminin receptor 1; *RPSA*: ribosomal protein SA; *BMPR1A*: bone morphogenetic protein receptor IA; *PTGS2*: prostaglandin-endoperoxide synthase 2; *VEGFA*: vascular endothelial growth factor A; *ADAMTS9*: a disintegrin-like and metalloprotease with thrombospondin type 1 motif 9; *CHL1*: cell adhesion molecule L1-like; *E2F1*: E2F transcription factor 1; *CYBB*: cytochrome b-245 heavy chain; *NOX2*: NADPH oxidase 2; *ESR1*: oestrogen receptor 1; *F2RL3*: coagulation factor II receptor-like 3; *MYBL1*: v-Myb avian myeloblastosis viral oncogene homologue-like 1; *NCOA2*: nuclear receptor coactivator 2; *P2RY1*: purinergic receptor P2Y, G-protein coupled, 1; *PF4*: platelet factor 4; *PLN*: phospholamban; *PPP2CA*: protein phosphatase 2, catalytic subunit,  $\alpha$ -isozyme; *TMOD3*: tropomodulin 3; *CXCL10*: interferon- $\gamma$ -inducible protein 10; *FCN3*: ficolin 3; *IGFBP7*: insulin-like growth factor-binding protein 7; *SCGB1A1*: secretoglobin 1A1; *SPP1*: secreted phosphoprotein 1 (osteopontin); *MMP1*: matrix metalloprotease 1; *MMP7*: matrix metalloprotease 7; *MMP13*: matrix metalloprotease 13; *EDNRB*: endothelin receptor B; *PTX3*: pentraxin 3; *CYP7B1*: cytochrome P450 7B1; *DAAM1*: Dishevelled-associated activator of morphogenesis 1; *RAC1*: Ras-related C3 botulinum toxin substrate 1; *RHOA*: Ras homologue A; *ROCK*: RHO kinase; *DSV*: Dishevelled; *WNT11*: Wingless member 11; *STAT1*: signal transducer and activator of transcription 1; *SMOC2*: SPARC-related modular calcium binding 2; *SFRP2*: secreted Frizzled-related protein 2; *COL3A1*: collagen IIIA1; *TNC*: tenascin C; *COL6A3*: collagen VIA3; *THBS2*: thrombospondin 2; *VWF*: von Willebrand factor; *CAV2*: caveolin 2; *MYCBP*: Myc binding protein; *FOS*: FBJ murine osteosarcoma viral oncogene homologue; *ANXA5*: annexin A5; *CYC1*: cytochrome C1; *GATA2*: GATA binding protein 2; *TGFBR1*: transforming growth factor- $\beta$  receptor I; *TGFBR2A*: transforming growth factor- $\beta$  receptor IIa; *GADD34*: growth arrest- and DNA damage-inducible protein 34; *HTR2B*: 5-hydroxytryptamine receptor 2B; *F2R*: coagulation factor II receptor; *ITPR1*: inositol trisphosphate receptor 1; *GREM1*: Gremlin; *LIMCH1*: LIM and calponin homology domains 1; *SLM2*: Sam68-like mammalian protein 2; *SPON1*: spondin 1; *ENPP2*: ectonucleotide pyrophosphatase/phosphodiesterase 2; *PRKY*: protein kinase, Y-linked, pseudogene; *MPEG1*: macrophage expressed 1; *TM2D1*: TM2 domain-containing 1; *MYO1B*: myosin IB; *BDNF*: brain-derived neurotrophic factor; *PODN*: podocan; *LOX*: lysyl oxidase; *FHL2*: four and a half LIM domains 2; *BKB2R*: bradykinin receptor B2; *PLK1*: Polo-like kinase 1; *PLK4*: Polo-like kinase 4; *ANLN*: anillin; *ANGPT1*: angiotensinogen 1; *DAPK1*: death-associated protein kinase 1; *SOD3*: superoxide dismutase 3; *ECGF1*: endothelial growth factor 1; *ADM*: adrenomedullin; *HVEM*: herpesvirus entry mediator; *DEFA1*: defensin  $\alpha$ 1; *DEFA3*: defensin  $\alpha$ 3; *S100P*: S100 calcium binding protein 3; *PROK2*: prokineticin 2; *MMP9*: matrix metalloprotease 9; *VEGF*: vascular endothelial growth factor; *EREG*: epiregulin; *IL8*: interleukin-8; *CYP1B1*: cytochrome P450 1B1; *ICAM1*: intercellular adhesion molecule 1; *IFNGR1*: interferon- $\gamma$  receptor I; *IL1B*: interleukin-1 $\beta$ ; *IL13RA1*: interleukin-13 receptor  $\alpha$ 1; *JAK2*: Janus kinase 2; *AIF1*: allograft inflammatory factor 1; *ALAS2*: 5'-aminolevulinate synthase 2; *TIMP2*: tissue inhibitor of metalloprotease 2; *IL7R*: interleukin-7 receptor; *ERAF*: erythroid differentiation associated factor; *AHSP*:  $\alpha$ -haemoglobin stabilising factor; *TCF7*: T-cell factor 7; *DSG2*: desmoglein 2; *RHOQ*: Ras homologue family member Q; *IGF*: insulin-like growth factor; *GTP*: guanosine triphosphate; *ECM*: extracellular matrix. <sup>#</sup>: Affymetrix Inc., Santa Clara, CA, USA; <sup>†</sup>: Agilent Technologies, Santa Clara, CA, USA; <sup>\*</sup>: Illumina Inc., San Diego, CA, USA; <sup>§</sup>: Exiqon A/S, Vedbæk, Denmark; <sup>f</sup>: Geniom Biochip MPEA *Homo sapiens* (Febit Biomed GmbH, Heidelberg, Germany).



for ion channel proteins, as compared to controls. They also identified genes distinguishing IPAH from FPAH. These included transforming growth factor- $\beta$  receptor III (*TGFRB3*), bone morphogenic protein 2 (*BMP2*), mitogen-activated protein kinase kinase 5 (*MAPKK5*), receptor for activated C kinase 1 (*RACK1*), apolipoprotein C-III (*APOC3*) and the gene encoding the ribosomal protein SA/laminin receptor 1 (*RPSA/LAMR1*), which were only found in the samples from patients with IPAH. In another study, a specific gene signature including genes relevant to regulation of actin-based motility, protein ubiquitination, and cAMP, transforming growth factor- $\beta$ , mitogen-activated protein kinase, oestrogen receptor, nitric oxide and platelet-derived growth factor (PDGF) signalling were identified in PAH as compared to healthy lung tissue. Importantly, bone morphogenic protein receptor II (*BMPR2*) expression was downregulated, even in subjects without a mutation in this gene [32]. Both of these studies [31, 32] bring to the light that not only mutations in the *BMPR2* gene, which are the most prominent cause of FPAH [58], but also downregulation of *BMPR2* in noncarrier patients could lead to PH pathogenesis. Additionally, females with IPAH displayed elevated expression of oestrogen receptor 1 (*ESR1*). Surprisingly, gene expression profiles of lung homogenates from PH-IPF patients resembled more those from normal controls than from PAH samples, indicating lack of a general PH signature [32]. Another microarray study of PAH samples focused on the metabolic heterogeneity. The gene encoding cytochrome P450 7B1 (*CYP7B1*), an isozyme for bile acid synthesis, was highly expressed in the PAH lung compared to controls [34]. Hsu *et al.* [33] compared gene expression profiles of systemic sclerosis (SSc)-associated pulmonary fibrosis and SSc-PAH subsets to IPF and IPAH. They detected genes involved in inflammation and activation of innate immunity such as *CXCL10* (interferon- $\gamma$ -inducible protein 10) as being upregulated in all groups independent of PH occurrence. In contrast to the aforementioned study, here, a specific PAH signature was detected that included genes involved in antigen presentation and chemokine activity. This signature was proportional to the degree of the PAH phenotype. Mura *et al.* [12] investigated IPF samples with and without co-existing PH. The detected PH gene signature was predominantly related to extracellular matrix (ECM) remodelling and PASMC and fibroblast proliferation/migration, whereas the non-PH phenotype was mostly associated with proinflammatory genes. Taken together, most of the gene expression studies of lung homogenate samples from PAH and lung fibrosis associated with PH revealed a common PH gene expression signature that included genes involved in cell proliferation, inflammation, immunity and ECM remodelling. The genes involved in inflammation are associated with PH; however, their involvement has equally been shown in non-PH samples, revealing a global influence of inflammatory genes rather than a PH signature.

#### *Laser capture microdissected pulmonary arteries*

In 2009, LAUMANN *et al.* [35] performed the first transcriptome-wide expression profiling of LCM resistance pulmonary vessels derived from explanted IPAH and nontransplanted donor lung tissues. The authors found elevated expression of planar cell polarity mediators such as Wingless member 11 (*WNT11*), Dishevelled (*DSV*) and RHO kinase (*ROCK*). This study pointed towards activation of developmental WNT signalling pathway in remodelling of intrapulmonary vessels. PATEL *et al.* [36] compared the gene expression of pulmonary arteries of IPF patients with and without coexisting PH to those of healthy donors, and revealed that mediators of PASMC and endothelial cell proliferation, WNT signalling, complement system activation and apoptosis were differentially expressed in IPF arterioles. The gene expression profiles of IPF and PH-IPF were similar, indicating comparable vascular changes in the IPF patient cohort with and without apparent PH. This finding could prompt the speculation that reprogramming of the transcriptome occurs before the appearance of PH. Comparison of gene expression profiles in intrapulmonary arteries of IPF and COPD with PH revealed that several genes belonging to the retinol metabolism and ECM pathways distinguishes the vascular remodelling of these two pulmonary diseases with PH [6]. Taken together, recent studies using LCM pulmonary arteries demonstrated that many identified genes were involved in WNT signalling, proliferation or ECM remodelling. LCM approaches highlighted that developmental signalling pathways are reactivated during vascular remodelling and that they could coordinate proliferative and ECM deposition processes.

#### *Isolated primary cells*

To date, several studies have investigated the mechanisms of proliferation, migration and contribution of single cell types to remodelling of the pulmonary artery wall. However, the molecular drivers of these processes remain largely unknown.

Even though endothelial dysfunction is a key feature in the pathogenesis of PAH [59], only one study so far analysed gene expression in human microvascular endothelial cells (HMVECs) by microarray analysis. COSTELLO *et al.* [38] compared gene expression profiles of primary HMVECs derived from lung tissue with those of cardiac origin and analysed gene expression upon hypoxic exposure. 90 genes were identified as being differentially regulated in the lung endothelium. Gremlin (*GREM1*) and *CXCR7* were verified as specifically upregulated in the lung cells in response to hypoxia. As *GREM1* is a bone morphogenetic

antagonist [60], this study pointed towards a regulatory relevance of *BMPR2* and the importance of this axis in PH. The identification of *CXCR7* emphasises the contribution of chemokines and their receptors to development of PH [38]. Currently, microarray studies comparing the gene expression profiles of healthy HMVECs and PH HMVECs are lacking. Therefore, the additional analysis of endothelial cells is necessary to give insight into gene expression underlying the observed endothelial dysfunction in PH. In line with this notion, in a recent study, RHODES *et al.* [61] investigated endothelial transcriptomes of healthy and IPAH patients using RNA sequencing, which presents an alternative or complementary approach to microarray analysis. A novel relationship between *BMPR2* dysfunction and reduced expression of endothelial collagen IV (*COL4*) and ephrin A1 (*EFNA1*) is proposed, which may underlie endothelial injury in PAH.

In contrast to endothelial cells, several studies have investigated gene expression profiles of human PSMCs. These cells constitute the primary cell type of the medial layer of the pulmonary vascular wall and are thought to contribute significantly to remodelling of the pulmonary artery. YU *et al.* [40] compared gene expression patterns in human PSMCs from IPAH and hereditary PAH (HPAH) to those of controls. Differentially expressed genes showed similar trends of expression in both HPAH and IPAH PSMCs. Many genes were involved in cellular growth/proliferation and regulation of the cell cycle. Additionally, certain vasoactive receptors such as bradykinin receptor B2 (*BKB2R*) were downregulated in both HPAH and IPAH cells. Apart from coding RNAs, noncoding RNAs have received increasing recognition but their involvement in remodelling processes is still elusive. In a recent study, GOU *et al.* [39] analysed miRNA gene expression in PSMCs and identified antiapoptotic miR-210 as the predominant miRNA induced by hypoxia. FANTOZZI *et al.* [37] examined whether BMP2 differentially regulates gene expression in PSMCs from normal subjects and IPAH patients. Exogenous administration of bone morphogenetic protein (BMP) might compensate for dysfunction of BMP signalling due to mutations in and/or downregulation of BMP receptors in IPAH patients. They observed that >1000 genes were oppositely regulated in IPAH PSMCs. The genes upregulated in IPAH cells included those associated with growth factors and ligands, membrane receptors, signal transduction proteins and kinases, transcription factors (e.g. c-Fos, c-Myc binding protein and nuclear factor  $\kappa$ B), and enzymes (e.g. leukotriene C4 synthetase and ATP synthase). The genes downregulated in IPAH cells were apoptotic inducers or proapoptotic factors, membrane receptors, ion channels or transporters, transcription factors (e.g. SKI, transcription factors E2F1 and TFE3, and breast cancer transcription factor ZABC1), and cytoplasmic enzymes and kinases. In summary, the regulation of growth and transcription factors point towards the involvement of PSMC proliferation, which leads to medial thickening and vascular remodelling driving aggravation and acceleration of the disease.

The most outer layer of the pulmonary artery (adventitia) is composed of fibroblasts [62] and inflammatory cells [63]. The contribution of fibroblasts to vascular remodelling is controversial [62, 64]. Although they proliferate upon diverse stimuli such as PDGF-BB [65] or hypoxia [66], the thickness of adventitia in pulmonary arteries from PH patients is not substantially different from that of vessels from healthy subjects [6, 62]. There is currently a very limited number of studies dealing with genes being regulated in the adventitial layer of PH. HSU *et al.* [33] compared gene expression not only in lung homogenates of IPAH and SSc-PAH but also in isolated primary fibroblasts from these patients. Fibroblasts derived from SSc-PAH and IPAH had a common signature (24 genes); however, several genes distinguished these two entities. Genes strongly upregulated in IPAH fibroblasts included chemokines and interleukins (ILs) such as IL-6, IL-8 and IL-13 receptor. This study emphasises the importance of inflammatory components in the adventitial layer, which indicates a possible role of these cells in PH pathogenesis.

### Circulating cells

In the last decade, numerous microarray studies have addressed mRNA and miRNA expression in circulating cells from PH patients (table 1). Except for one, all these studies analysed circulating peripheral blood mononuclear cells (PBMCs), which include multiple cell types such as lymphocytes, monocytes and natural killer cells.

In 2004, BULL *et al.* [41] compared gene expression of PBMCs from IPAH and secondary PAH (sPAH) (including portal hypertension, calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, interstitial lung disease, exposure to the anorexigens phentermine/fenfluramine and chronic pulmonary thromboembolic disease) patients with controls, and found the gene ontology classes "inflammatory response" and "response to stress" to be modulated in IPAH compared to healthy controls. IPAH and sPAH had similar expression profiles. Similarly, ULRICH *et al.* [42] found genes involved in inflammatory mechanisms, host defense or endothelial function were affected in IPAH PBMCs. A study comparing PBMCs from SSc-PAH to IPAH PBMCs indicated that PBMC gene expression was similar in both disease groups and correlated with known predictors of survival in PAH. This study additionally supports the notion that angiogenesis and chemotaxis/inflammation are associated with severity of PAH [43]. A study



in 2010 by PENDERGRASS *et al.* [45] investigated whether the coexistence of PAH in limited systemic sclerosis (ISSc) influenced gene expression in PBMCs. Gene expression analysis distinguished ISSc samples from healthy controls and separated ISSc-PAH from ISSc without PAH, pointing towards a specific PAH signature. CHEADLE *et al.* [47] analysed transcript profiles of PBMCs from IPAH and SS-PAH as compared to healthy donors. Multiple gene expression signatures were found that distinguished the various disease groups from controls. One of these gene sets, erythrocyte maturation, was enriched specifically in PAH and correlated with haemodynamic measures of increasing disease severity in patients with IPAH. RISBANO *et al.* [46] compared gene expression profiles of PBMCs from SS patients with and without coexisting PAH, and identified IL-7 receptor (*IL7R*) to be significantly decreased in samples from PAH patients. CHESNÉ *et al.* [48] compared PBMC gene expression from healthy controls, PAH and cystic fibrosis patients in order to identify an end-stage chronic respiratory disease-related gene signature. Microarray results were validated in a second independent cohort and COPD patients were added to validate the common signature. In the common signature group, T-cell factor 7 (*TCF7*) and *IL7R*, two genes related to T-lymphocyte activation, were found to be downregulated. Taken together, two studies emphasise the importance of *IL7R* as a potential biomarker in PH. HEMNES *et al.* [13] compared PBMCs from vasodilator-responsive (VR)-PAH to vasodilator-nonresponsive PAH patients. Differences in gene expression patterns on microarray analysis included cell-cell adhesion factors, and cytoskeletal and Rho GTPase genes. *DSG2*, encoding a desmosomal cadherin involved in WNT/ $\beta$ -catenin signalling, and Ras homologue family member Q (*RHOQ*), which encodes a cytoskeletal protein involved in insulin-mediated signalling, were sufficient to correctly distinguish the five VR-PAH patients in the validation cohort from the rest of the cohort, implying prognostic potential of these genes.

In circulating PBMCs, miRNA profiles also have been analysed. RHODES *et al.* [11] showed that miR-150 was downregulated in the plasma of IPAH patients and correlated with the 2-year survival of these patients. Another study of PBMCs from PH patients compared healthy to moderate (mean mean pulmonary artery pressure (mPAP) 29 mmHg) and severe (mean mPAP 46 mmHg) PH, and detected several miRNAs as being downregulated (miR-451 and miR-1246) or upregulated (miR-23b, miR-130a and miR-191), and whose expression levels were proportional to the degree of PH [17]. A recent study by SARRION *et al.* [49] highlighted the potential relevance of miR-23a in IPAH as it correlated with the patient's pulmonary function and might therefore serve as a potential biomarker.

To date, there has only been a single study analysing a subpopulation of PBMCs, namely lymphocytes. WEST *et al.* [44] generated immortalised lymphocytes from FPAH patients with *BMPR2* mutations as compared to mutation positive but disease-free family members, and subjected the resulting lymphoblastoid cell lines to microarray analysis. Disease status in *BMPR2* mutation carriers correlated with differential gene expression in proliferation, GTP signalling and stress response pathways. The oestrogen metabolism gene *CYP1B1* (cytochrome P450 1B1) was found to be significantly downregulated in female PAH patients. The low expression of this gene was associated with disease severity. Importantly, this study illustrates that *BMPR2* mutations influence inflammatory cell gene expression profiles. In the future, further microarray analyses of PBMC subpopulations should be conducted to get deeper insight into alterations of gene profiles in specific populations and to delineate their individual contribution to PH. Additionally, expression profiles of other cell types found in the blood, such as polymorphonuclear neutrophils, have not been analysed so far. This may, however, be of relevance, as the neutrophil to lymphocyte ratio is reported to be significantly increased in patients with PAH [67].

Taken together, the easy accessibility and identification of several proposed circulating markers such as miRNAs (particularly miR-23) provide an opportunity to apply them as prognostic tools for IPAH or PH associated with lung diseases and determination of disease progression or therapy evaluation. However, detected differentially regulated gene signatures in disease conditions have to be validated in a separate cohort to confirm prognostic potential. This has so far only been performed in a very limited number of studies. Experience from cancer research has demonstrated how essential such verifications are for the approval of reliable biomarkers [68].

### Summary of microarray studies on rodent lung tissue

In parallel to studies on human samples, several microarray studies have been performed on hypoxia- and monocrotaline (MCT)-induced PH in rodents. Although animal models may recapitulate only some aspects of human vascular remodelling, such as PASM proliferation or inflammation, they represent an excellent tool to investigate not only end-stage disease, but also disease onset, progression or reversal.

The classical and well accepted model for mild PH is hypoxia-induced PH in rats and mice [69]. Investigators have concentrated on diverse disease stages and analysed gene expression at different time-points of disease development (short hypoxia exposure [70, 71]), end-stage disease (long-term hypoxia exposure [50, 72]) or reverse remodelling (reoxygenation [73, 74]). Cumulatively, these studies

revealed that during disease onset, multiple transcription and growth factors, such as endothelin (*EDN1*) [70] and neurotrophic tyrosine kinase receptor type 2 (*NTRK2*) [71], are activated. In end-stage disease, marked structural changes have been observed, such as in procollagen, tenascin C (*TNC*), S100 calcium-binding protein A4 (*S100A4*), cluster of differentiation 36 (*CD36*) and FK506 binding protein 1A (*FKBP1A*) [50]. The reverse remodelling approaches can shed the light on genes that are involved in reverse remodelling processes, which include adenosylmethionine decarboxylase 1 (*AMD1*) [74], Ras GTPase-activating-like protein (*IQGAP1*), insulin-like growth factor-binding protein 3 (*IGFBP3*) and lactoferrin (*LTF*) [73].

The inflammatory component of human PH is best resembled by the MCT-induced PH model in rats [75]. In this PH model, numerous studies analysed mRNA or miRNA expression changes and revealed alterations in genes involved in response to wounding and inflammation, blood vessel morphogenesis and genes encoding proteases [76–78], as well as miR-22, miR-30, let-7f, miR-322 and miR-451, miR-21, and let-7a [79].

Additionally, diverse studies have examined the influence of PH medication testing [80], strain differences [81], intermittent or sustained exposure to hypoxia [82] and sex differences [83] on the gene expression changes during development of PH in various rodent models.

### Conclusions, limitations and outlook

To date, more than 25 human microarray studies supplying enormous amounts of gene expression data, ranging from single gene expression to complex pathway analyses, have been performed. Over the last 15 years, more easily accessible compartments, such as circulating cells, have been investigated more frequently (figure 1).

Gene expression changes in the ECM, angiogenesis, inflammatory processes and the WNT pathway are now increasingly recognised to be important for processes underlying PH (table 1 and figure 2). The direct link between WNT signalling and developmental processes, cell proliferation and migration [84] points towards its contribution to vascular remodelling. Although individual fingerprints are observed for diverse cell types and compartments, many of the regulated pathways are shared. This indicates that similar signalling patterns are involved in PH pathogenesis in different pulmonary compartments (figure 2). Whether these are adaptive or pathological responses and what the precise mechanisms and kinetics of their regulation are has yet to be elucidated. Further understanding of the underlying mechanisms could help to develop effective treatment options for patients with PAH and PH associated with CLD.

Each of the studies discussed has concentrated on a certain aspect of disease pathogenesis. It can be envisioned that the identification of common signalling signatures and local gene regulatory networks could lead to detection of important regulators governing more fundamental biological processes and pathways or specific parts of it, which are involved in the pathogenesis of PH. Moreover, vast amounts of data remain to be explored. The lack of consistency in sample collection, storage and preparation as well as microarray platform and bioinformatic analysis hampers direct comparisons and prevents general conclusions. However, the available data can still be a valuable source for the generation of new hypotheses

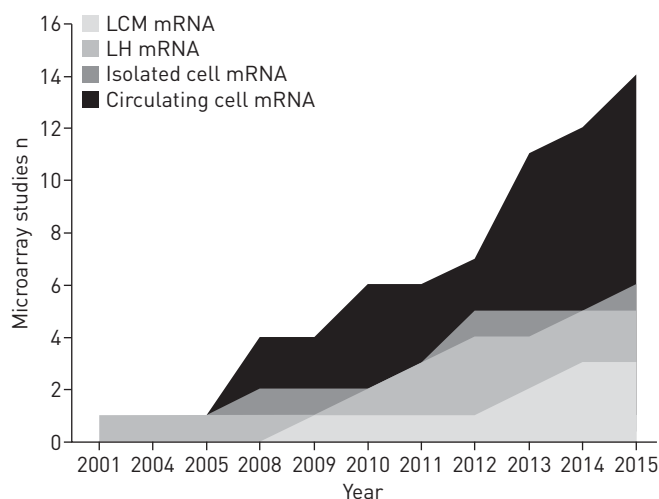


FIGURE 1 The number of human microarray studies published since 2001 on lung homogenate (LH), laser capture microdissection (LCM), and isolated or circulating cells with a focus on pulmonary arterial hypertension or pulmonary hypertension associated with chronic lung diseases.

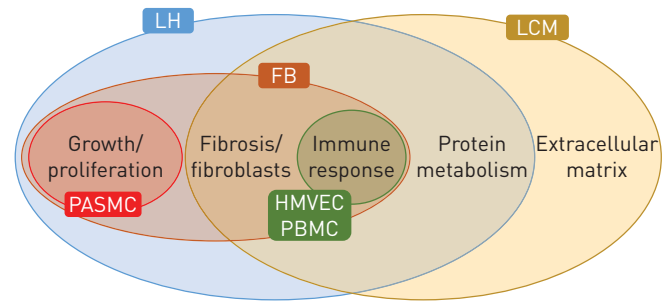


FIGURE 2 Venn diagram depicting overlapping and diverging microarray analysis results of the discussed studies. LH: lung homogenate, LCM: laser capture microdissection, FB: isolated fibroblast, PASM: pulmonary artery smooth muscle cell; HMVEC: human microvascular endothelial cell; PBMC: peripheral blood mononuclear cell.

about the molecular mechanisms driving PH. The utility of microarray results could be enhanced by standardisation of data acquisition and analysis, and thus hold the promise that they may significantly improve our understanding of PH pathomechanisms.

In the near future, new technologies will complement microarray platform. Exemplarily, next-generation RNA sequencing will be applied to reveal a snapshot of RNA presence and quantity, alternative splicing, post-transcriptional modifications, gene fusion, and mutations/single-nucleotide polymorphisms. Therefore, it will help to yield a more holistic impression of the disease pathogenesis [85]. Indeed, a recent study already utilised this technology to investigate endothelial transcriptomes of IPAH patients [61].

In summary, in future RNA expression studies, it will be important to 1) compare and harmonise gene expression from animal models and human disease, 2) use system biology approaches in order to identify complex disease development signatures and to understand the dynamics of disease development on the level of regulatory networks, and 3) analyse noncoding gene expression that could influence coding gene expression.

### Acknowledgement

We would like to thank Leigh Marsh (Ludwig Boltzmann Institute for Lung Vascular Research, Graz, Austria) for critical reading of the manuscript.

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