

# Gene expression profile in persistent pulmonary hypertension of newborn, preliminary report

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## Abstract

**Background:** Persistent pulmonary hypertension of the newborn (PPHN) is a serious condition with high morbidity and mortality. The therapeutic approach, even today, represents a major challenge. There is considerable interest in the understanding of the signaling pathways that regulate vasoconstriction and pulmonary vascular remodeling.

**Objective:** We set out to identify the expression of genes S100A9 and solutes transporter, as well as their participation in the physiopathological events of PPHN.

**Design and methods:** This is a cross-sectional study that focuses mainly on up- and down-regulated genes involved in endothelial metabolism, hypoxia transporters, ionic and nucleotides metabolism. The total RNA was obtained from blood samples of healthy full-term newborns (negative controls) and patients with PPHN. The preparation of samples for microarray analysis was as follows: control samples were stained with fluorescent nucleotides dUTP-Cy3 and pathological samples with dUTP-Cy5; samples were co-hybridized, a microarray analysis was performed using an Array Scanner Packard. Additionally, reverse transcriptase polymerase chain reaction (RT-PCR) for the genes S100A9 and solutes transporter was done. Comparative and descriptive analyses were done using t-test for independent variables, considering a  $p < 0.05$  as significant. The elements with a z-score of more than 2 standard deviations are genes likely to be differentially expressed.

**Results:** From the total (10,000) genes analyzed, 364 (3.64%) were differentially regulated in their expression; those that registered an up-regulation were 1.13%, while 2.51% were down-regulated. S100 proteins type A9 and A12 were the most over-expressed, as well as Rho family GTPase 3. Down-regulation in the hypoxia-inducible factor 1 (HIF-1) and HIF-1 alpha subunit inhibitor was found. S100A9 and RSC1A1 had increased mRNA expression under PPHN conditions compared to healthy newborn infants.

**Conclusions:** The up-regulation from both genes could explain, in part, the vasoreactivity and vascular remodeling characteristic of PPHN. A deeper understanding of the pathophysiology of PPHN could have a positive impact on the development of specific therapies.

## Keywords

Persistent pulmonary hypertension of newborn, gene S100A9, solutes transporter, RT-PCR.

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## Introduction

During fetal life, pulmonary vascular resistance (PVR) is physiologically increased; at birth, PVR progressively decreases, allowing changes in the flow through the ductus arteriosus and foramen ovale, which eventually close [1]. As a result, pulmonary blood flow rises approximately 8 to 10 times. At birth, these changes may fail, and pulmonary pressures remain abnormally high. This results in persistent pulmonary hypertension of the newborn (PPHN) [2] (in the past it was also called persistence of fetal circulation), that has a reported incidence of 0.46-6.8/1,000 live births in developed countries [3].

PPHN occurs when the PVR remains abnormally high and results in low pulmonary flow; the criteria for PPHN diagnosis are based on clinical (need for respiratory support because of a > 10% difference between pre-ductal and post-ductal saturation, more than 2 episodes of desaturation < 85% over a 12-hour period of clinical observation and notwithstanding underlying lung disease optimal medical treatment) as well as echocardiographic features (structurally normal heart; peak gradient of systolic pulmonary arterial pressure > 35 mmHg, estimated pulmonary arterial pressure greater than 2/3 of the systemic arterial pressure and presence of right-to-left shunt across a patent foramen ovale and/or patent ductus arteriosus). Echocardiography is the gold standard for diagnosis [4].

PPHN has a multitude of etiologies, including abnormally constricted pulmonary vasculature caused by parenchymal diseases (pneumonia, meconium aspiration syndrome, sepsis), hypoplastic pulmonary vasculature and normal parenchyma with remodeled pulmonary vasculature [5].

Inhaled nitric oxide (iNO) is the gold standard therapeutic for the PPHN; other strategies such as sildenafil, milrinone, and endothelin-1 blockers have been suggested [3]. However, no encouraging results in improving the mortality rate [6-9], estimated at 10-20%, and suggested to be higher in developing countries, have been shown [5]. Therefore, it is a challenge to find signaling pathways that regulate pulmonary vasoconstriction and vascular remodeling, in order to find new therapeutic opportunities. Thus, the main objective of this preliminary report was to identify changes in the gene expressions in patients with PPHN, focusing on those genes related with this physiopathology and mainly involved in endothelial metabolism, energetic disturbances and cellular proliferation, amplification of the proinflammatory cascade and particularly on the S100A9 gene, as well as some ionic transporters.

## Material and methods

### *Design and methods*

A cross-sectional study was performed at the Neonatal Intensive Care Unit of the Pediatric Hospital, Western National Medical Center, Guadalajara, Jalisco, México. The Research and Ethics committee of the Hospital approved this

study. Written informed consent was obtained from the parents of each patient before enrollment.

### Selection of patients

Full-term neonates were enrolled in the study if they met the following criteria:

1. Study group: mechanical ventilatory support, fraction of inspired oxygen > 0.50, PPHN confirmed by echocardiogram Doppler, which shows the jet of blood flow that escapes through the tricuspid valve; the jet speed is a direct indicator of right ventricular pressure and an indirect indicator of pulmonary artery pressure, based on the Bernoulli equation (the pressure gradient between two cameras through a narrow orifice is equal to four times the speed squared); we consider PPHN when it was superior to 30 mmHg [10]. A sample of 1 milliliter of blood was obtained through the umbilical arterial catheter from each infant (n = 12).

2. The control group included 12 healthy full-term newborn infants from Gynecology and Obstetrics Hospital, Western National Medical Center, Guadalajara, Jalisco, México, that were delivered by elective cesarean section, had normal weight, Apgar score above 7 at 1 and 5 minutes and without oxygen requirements at any time. A sample of 1 milliliter of blood was obtained from the umbilical cord from each infant in the delivery room.

### Molecular biological studies

#### Extraction and quantification of total RNA

Total mRNA was isolated from white blood cells using TRIzol reagent® (Invitrogen), following the guanidine isothiocyanate method [11]. Chloroform was added and the RNA was precipitated from the resulting aqueous phase with

isopropanol at 4°C overnight. Only RNA samples with a 260/280 nm absorption ratio between 1.8 and 2.0 were used for further experiments. The integrity of the RNA samples was tested using standard RNA gel analysis [11, 12].

#### RT-PCR analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out as described previously [13]. A semi-quantitative PCR method was established based on the amplification of the target gene and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as control and to adjust the mRNA concentration. Aliquots of 2 µg of total RNA were used for reverse transcription in 0.05 M Tris-HCl buffer (pH 8.3) containing 40 mM KCl, 7 mM MgCl<sub>2</sub>, 0.05 U/ml RNase inhibitor and 200 IU Maloney's murine leukemia virus (M-MLV) reverse transcriptase. Samples were first incubated at 70°C for 10 min and then at 37.5°C for a further 60 min. Finally, the reverse transcriptase was inactivated by maintaining the samples at 95°C for 10 min. The cDNA obtained for PCR was either used immediately or stored at -20°C. Unless otherwise stated, gene amplification was performed in a PCR buffer of 50 mM Tris-HCl (pH 9.0) and 50 mM NaCl, containing a mix of 100 M dNTPs and 1 U of Taq DNA polymerase. Each amplification cycle was of: 95°C for 1 min; 60°C for 1 min; 72°C for 1.5 min and a final extension at 72°C for 5 min. The primers used to amplify the mRNAs were as shown in **Tab. 1**.

PCR-products were analyzed by 2.5% agarose gel electrophoresis. The intensity of the bands was determined using a video gel documentation and analysis system (Chemic Doc XRS, BioRad), and evaluated semi-quantitatively by densitometry. The density of the PCR products was normalized to the corresponding amplification of the internal

**Table 1.** The primers used to amplify the mRNAs.

Gene	Upper primer, base position	Primers	GenBank ID	Amplified a PCR product (bp)
S100A9	71	5'- CGC AAC ATA GAG ACC ATC ATC AAC ACC -3'	NM_002965.3	230
	300	5'- AGC CTC GCC ATC AGC ATG ATG AAC TCC -3'		
RSC1A1	1321	5'- CAA AGA GCC TGG TGA AGA CAG CCA CT -3'	NM_006511.1	468
	1788	5'- GCA GGA AGA AGT CCT TGT TGG CAT AG -3'		
GAPDH	11	5'- CGC TTC GCT CTC TGC TCC TCC TGC TC -3'	NM_001256799	600
	611	5'- GGG GTG CTA AGC AGT TGG TGG TGC AG -3'		

bp: base pair.

GAPDH gene. The results were expressed as relative intensity in arbitrary units compared to the control values and the data represent the mean of at least four independent experiments, each performed in duplicate.

#### Microarray analysis

The preparation of samples for microarray analysis was as follows: for each control sample, RNA (10 µg) was stained with dUTP-Cy3, and the pathological sample was stained with dUTP-Cy5. Samples were co-hybridized on the microarray according to the method described by Richmond et al. [14]. Plates of high-density microarrays containing stains were prepared according to the method described by Tao et al. [15]. Microarrays were analyzed with microarray scan Array Scanner Packard. The resulting images were analyzed by the average density of pixels (intensity) for each spot of the array, which was used for the detection of quantarray program [16].

#### Data analysis

Statistical analyses were performed with the program GenArise (National Autonomous University of Mexico, Institute of Cellular Physiology, Unit of Microarrays) [16]. The goal of GenArise is to identify genes that are candidates to be differentially expressed. GenArise performs several transformations: background correction, lowest normalization, intensity filter, replicate analysis, and selection of differentially expressed genes. The software identifies differentially expressed genes by calculating an intensity-dependent z-score. It uses a sliding window algorithm to calculate the mean and standard deviation within a window surrounding each data point, and it defines a z-score where z measures the number of standard deviations a data point is from the mean. Applying these criteria, the elements with a z-score of more than 2 standard deviations are genes likely to be differentially expressed [16].

On this base, we calculated the percentage of frequency of those genes associated with different metabolic and structural functions and we compared them with those that were down-regulated.

In the cases of gene validation, comparative analyses were done using t-test for independent variables considering a least  $p < 0.05$  as significant.

## Results

### Microarray analysis

Blood samples obtained from healthy newborn infants and patients with PPHN were analyzed using the GenArise system for microarrays. A platform of 10,000 genes was used to determine the significant changes in the gene expression, considering a least a z-score from +2 to +4.28 for up-expression or -2 to -5.1 for down-expression. Results showed that from the total gene analyzed, only 364 (3.64%) genes were differentially regulated in their expression. Those that registered an up-regulation were 1.13%, while 2.51% were down-regulated in their expression.

### Genes up-regulated in the PPHN

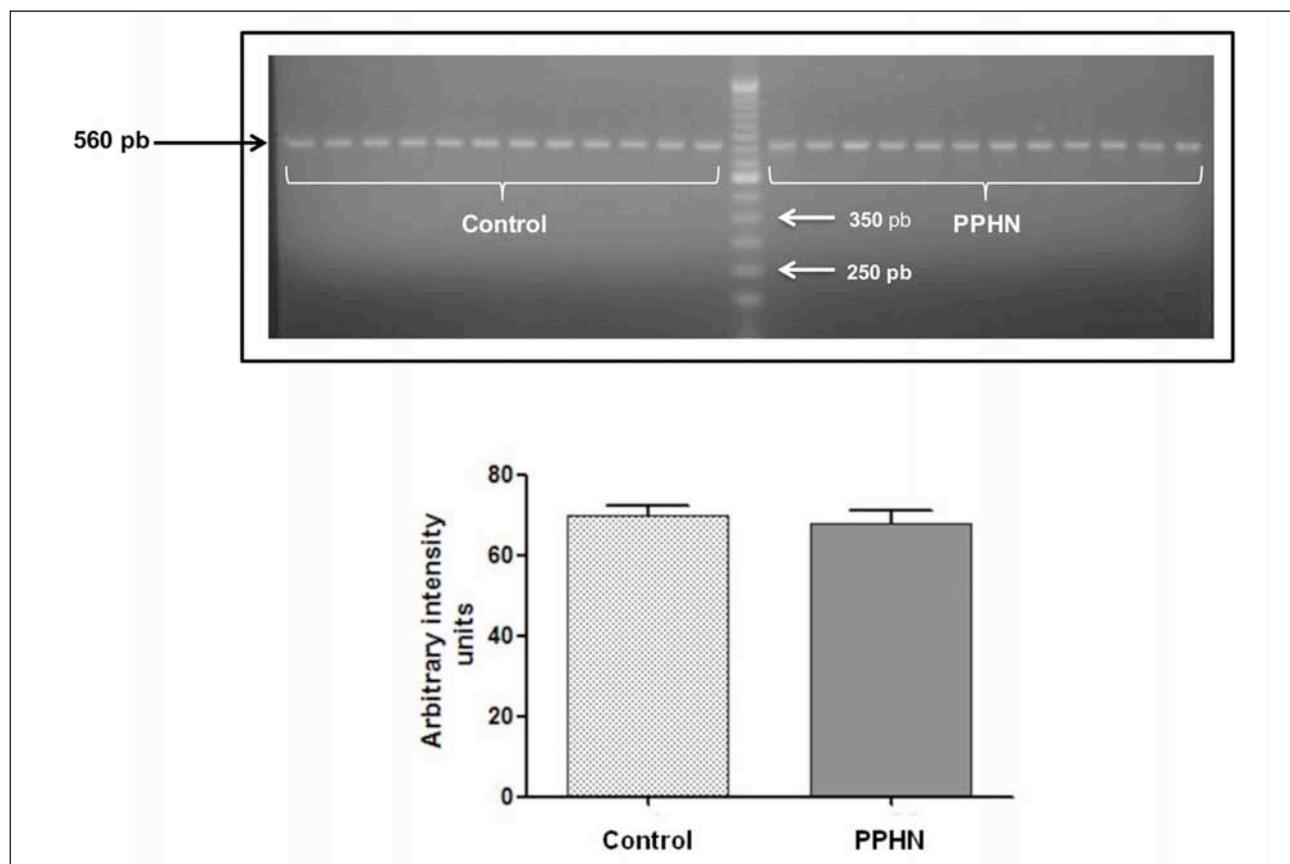
According to the results obtained from the microarray analyses, the 1.13% genes was observed as over-expressed in PPHN; this percentage was taken as 100%.

The genes majorly expressed were those related to protein kinases and central nervous system in 7.1% (8 genes) and 6.2% (7 genes), respectively, in relation to those down-regulated (**Fig. 1**). Those genes associated with oxidative stress and cell cycle were expressed in a frequency of 4.4 (5 genes) 3.5 (4 genes), respectively, and metalloproteinase 2.1 (15 genes). All of them were mainly increased in relation to those down-regulated (**Fig. 1**). Additionally, genes related to immunity (4% [7 genes]) and transferases (2.5% [3 genes]) were observed to be down-regulated (**Fig. 1**).

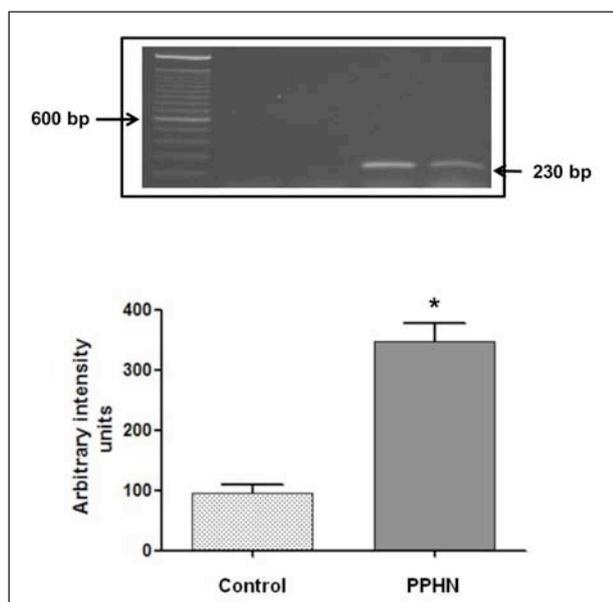
On this base, we focused mainly on those genes related to the physiopathology of PPHN, selecting the most representative and with high z-score values. It permitted us to make a better and integral description associated with its metabolic function. Thus, we found that genes involved in endothelial metabolism and hypoxia were in high percentage of frequency up-regulated (**Fig. 2**), whereas those related to ionic transporters and nucleotides metabolism were mainly down-regulated (**Fig. 2**).

### Genes involved with the endothelial metabolism in the PPHN

*Up-regulated genes.* Results showed that under PPHN conditions, S100 proteins type A9 and A12 were the most over-expressed (**Tab. 2**). This protein family contains into their structure 2 EF-hand calcium-binding motifs; they are localized



**Figure 1.** From the complete microarrays analysis, all the genes related with the persistent pulmonary hypertension of newborn (PPHN) disease, such as the metabolisms of endothelial, ionic transport, GTP and hypoxia were identified; 58 up-regulated genes and 26 down-regulated genes in the metabolic pathways were found.



**Figure 2.** The upper panel shows the S100A9 mRNA expression of two representative samples corresponding to the control and persistent pulmonary hypertension of newborn (PPHN) groups. The lower panel represents a semi-quantitative analysis of S100A9 expression. Data represents a mean  $\pm$  standard deviation from 12 duplicated samples. Statistically significant with respect to the control at  $p < 0.05$ .

in the cytoplasm and/or nucleus of a wide range of cells and involved in the regulation of a number of cellular processes, such as cell cycle progression and differentiation. S100A9 protein may function in the inhibition of casein kinase and altered expression of this protein is associated with the disease cystic fibrosis, whereas S100A12 is proposed to be involved in specific calcium-dependent signal transduction pathways and its regulatory effect on cytoskeletal components may modulate various neutrophil activities.

*Down-regulated genes.* Under PPHN conditions, trophinin and collagen type XVIII, alpha1 were mainly down-expressed (**Tab. 2**). Thus, trophinin gene encodes a membrane protein that mediates apical cell adhesion. The proteolytically produced C-terminal fragment of type XVIII collagen is endostatin, a potent antiangiogenic protein.

#### Genes involved with hypoxia in PPHN

*Up-regulated genes.* Part of the genes over-expressed in the PPHN are the surfactant protein D,

**Table 2.** Up-regulation and down-regulation expression of genes involved in endothelial metabolism.

	Fold change	Gene name	GenBank ID	Synonyms
Up-regulation	4.3	S100 calcium binding protein A9 (calgranulin B)	NM_002965	60B8AG, CAGB, CFAG, CGLB, L1AG, LIAG, MAC387, MIF, MRP14, NIF, P14
	3.1	S100 calcium binding protein A12 (calgranulin C)	NM_005621	CAAF1, CGRP, ENRAGE, MRP6, p6
Down-regulation	-3.8	Trophinin	NM_016157	RP6-14C6.1, KIAA1114, MAGE-d3, MAGED3
	-3.7	Collagen, type XVIII, alpha 1	NM_030582	FLJ27325, KNO, MGC74745
	-2.3	Vascular endothelial growth factor-related protein	NM_002632	PLGF, PIGF-2

a protein involved in immunity, and the hypoxia-inducible factor 1 (HIF-1), a key regulator of the hypoxic response and angiogenesis (**Tab. 3**).

Ras-related guanosine triphosphate binding D is a monomeric guanine nucleotide-binding protein. Those proteins act as molecular switches in numerous cell processes and signaling pathways.

The ferritin, heavy polypeptide 1 gene (**Tab. 3**), is a gene that encodes the heavy subunit of the ferritin protein. It constitutes the major intracellular iron storage. A major function of ferritin is the storage of iron in a soluble and nontoxic state. Variation in ferritin subunit composition may affect the rates of iron uptake and release in different tissues (**Tab. 3**).

*Down-regulated genes.* Regarding the genes down-expressed in PPHN, we found the down-regulation in the HIF-1 and HIF-1 alpha subunit inhibitor, a key regulator of the hypoxic response and angiogenesis, as well as different types of chains for hemoglobin, delta, zeta, epsilon 1 and theta. Two alpha chains plus two beta chains constitute adult hemoglobin (**Tab. 3**).

#### Genes involved with the ionic transporters in the PPHN

*Up-regulated genes.* In the patients with PPHN, it was possible to find several over-expressed genes involved with the ions transport, such as the following ones.

- Solute carrier family 26: this gene is primarily expressed in the liver, pancreas, and brain (**Tab. 4**).
- Gap junction gene: this gene allows facilitated diffusion (by an energy-independent process) by passage through a transmembrane aqueous pore or channel without a carrier-mediated mechanism (**Tab. 4**).

- Matrix metalloproteinase 9: gene described as interacting selectively with calcium ions ( $\text{Ca}^{2+}$ ).
- ATPase,  $\text{Na}^+/\text{K}^+$  transporting: it is an integral membrane gene responsible for establishing and maintaining the electrochemical gradients of Na and K ions across the plasma membrane (**Tab. 4**).

*Down-regulated genes.* Arrays results showed that around 20 genes were found down-expressed (**Tab. 5**). We highlight some of them.

- Amiloride-sensitive cation channel 3: encoded by this gene is an acid sensor and may play an important role in the detection of lasting pH changes.
- Arginine vasopressin receptor 2 (V2 receptor): it is expressed in the kidney tubule, predominantly in the distal tubule and collecting ducts, where its primary property is stimulating mechanisms that concentrate the urine and maintain water homeostasis in the organism.
- Solute carrier family 28 (sodium-coupled nucleoside transporter): this gene interacts selectively with a nucleoside, aquaporin 6, kidney-specific; this gene is an aquaporin protein, which functions as a water channel in cells.
- Cholinergic receptor, nicotinic, alpha 1 (muscle: this gene encodes an alpha subunit that plays a role in acetylcholine binding/channel gating) (**Tab. 5**).

#### Genes involved with the nucleotides metabolism in the PPHN

It was interesting to observe that under PPHN conditions several genes related to nucleotides as GTP and ATP function appear with changes in their expression (**Tab. 6**). Here, we only mention some of them.

**Table 3.** Up-regulation and down-regulation of genes involved in hypoxia.

	Fold change	Gene name	GenBank ID	Synonyms
Up-regulation	2.4	Surfactant protein D	NM_003019	COLEC7, PSP-D, SFTP4, SP-D
	2.2	Ferritin, heavy polypeptide 1	NM_002032	FTH, FTHL6, MGC104426, PIG15, PLIF
Down-regulation	-5	Hemoglobin, delta	NM_000519	
	-4.4	Hemoglobin, zeta	NM_005332	
	-4.1	Hemoglobin, epsilon 1	NM_005330	
	-3.7	Hemoglobin, theta 1	NM_005331	
	-2.1	Hypoxia-inducible factor 1, alpha subunit inhibitor	NM_017902	DKFZp762F1811, FIH1, FLJ20615, FLJ22027

**Table 4.** Up-regulation metabolism involved with ionic transporters.

	Fold change	Gene name	GenBank ID	Synonyms
Up-regulation	3.8	Solute carrier family 26 (sulfate transporter), member 1	NM_022042	EDM4, SAT-1, SAT1
	3.4	Regulatory solute carrier protein, family 1, member 1	NM_006511	RS1
	3	Potassium voltage-gated channel, shaker-related subfamily, member 1	NM_000217	AEMK, EA1, HUK1, KV1.1, MBK1, MGC126782, MGC138385, MK1, RBK1
	2.1	Gap junction protein, alpha 8, 50kDa (connexin 50)	NM_005267	CAE, CAE1, CX50, CZP1, MP70
	2	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, beta 2 polypeptide	NM_001678	AMOG
	2	Potassium voltage-gated channel, subfamily F, member 1	NM_002236	IK8, KCNF, KV5.1, MGC33316, kH1

- Guanylate cyclase 2D.
- Myosin head domain containing 1: these genes interact selectively with ATP, adenosine 5'-triphosphate, a universally important co-enzyme and enzyme regulator.
- Rho family GTPase 3: this gene regulates the organization of the actin cytoskeleton in response to extracellular growth factors.
- Chimerin (chimaerin) 1: this gene increases the activity of a GTPase, an enzyme that catalyzes the hydrolysis of GTP (**Tab. 6**).

**Fig. 2** and **Fig. 3** show the protein S100A9 and the RSC1A1, which were increased in the mRNA expression level under PPHN conditions compared to healthy newborn infants.

## Discussion

PPHN is characterized by hyperreactivity of the muscle layer in pulmonary arterioles [3, 17]. Endothelial cell dysfunction is most likely reflected in increased vasoconstriction [18,

19]. The pathogenesis of PPHN is complex and multifactorial, often resulting from the interaction between genetic susceptibility and environmental or acquired factors, including hypoxia, oxidative and hemodynamic stress, inflammation, and altered expression of vaso-active products [3, 18].

Despite important discoveries concerning vascular biology, there are gaps related to vasoreactivity, proliferative vessel growth and vascular remodeling, which are all characteristics of PPHN [20]. In this study, gene expression in the pathogenesis of PPHN was grouped in endothelial metabolism, hypoxia factors, ionic channels and nucleotides metabolism. Those are all important metabolic pathways that contribute to the cellular behavior in this pulmonary vascular disease.

### *Endothelial metabolism*

In newborns with PPHN, we found an over-expression of S100A9 and S100A12 proteins, which belong to the S100 family [21]. The first one

**Table 5.** Down-regulation metabolism involved with ionic transporters.

	Fold change	Gene name	GenBank ID	Synonyms
Down-regulation	-3.6	Amiloride-sensitive cation channel 3	NM_020322	ASIC3, SLNAC1, TNaC1
	-2.8	Arginine vasopressin receptor 2 (nephrogenic diabetes insipidus)	NM_000054	ADHR, DI1, DIR, DIR3, MGC126533, MGC138386
	-2.8	Potassium channel, subfamily K, member 15	NM_022358	KCNK11, KCNK14, KIAA0237, KT3.3, TASK-5, TASK5
	-2.8	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2	NM_001046	BSC, BSC2, MGC104233, NKCC1
	-2.6	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 1	NM_004213	CNT1, HCNT1
	-2.6	Aquaporin 6, kidney-specific	NM_001652	AQP2L, KID
	-2.5	Solute carrier family 25 (mitochondrial carrier, brain), member 14	NM_022810	RP1-20I3.5, BMCP1, UCP5
	-2.3	Solute carrier family 2 (facilitated glucose transporter), member 8	NM_014580	GLUT8, GLUTX1
	-2.1	Cholinergic receptor, nicotinic, alpha 1 (muscle)	NM_000079	ACHRA, ACHRD, CHRNA, CMS2A, FCCMS, SCCMS

**Table 6.** Up-regulation involved with nucleotides metabolism.

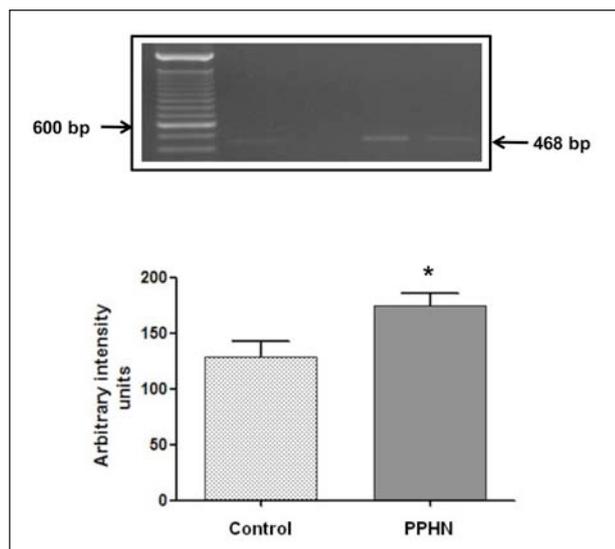
	Fold change	Gene name	GenBank ID	Synonyms
Up-regulation	2.6	Guanylate cyclase 2D, membrane (retina-specific)	NM_000180	CORD6, CYGD, GUC1A4, GUC2D, LCA, ROS-GC1
	2.5	Myosin head domain containing 1	NM_025109	FLJ22865
	2.4	Rho family GTPase 3	NM_005168	ARHE, Rho8, RhoE, memB
	2.4	Chimerin (chimaerin) 1	NM_001822	ARHGAP2, CHN, RHOGAP2
	2.3	Rho GTPase activating protein 11A	NM_014783	GAP (1-12), KIAA0013, MGC70740
	2.3	RAB14, member RAS oncogene family	NM_016322	RP11-165P4.4, FBP, RAB-14
	2.3	Ras-related GTP binding D	NM_021244	DKFZP761H.71, RAGD, bA11D8.2.1
	2.2	Ribosomal protein S6 kinase, 90kDa, polypeptide 2	NM_021135	HU-2, MAPKAPK1C, RSK, RSK3, S6K-alpha
	2.2	CDC42 effector protein (Rho GTPase binding) 3	NM_006449	BORG2, CEP3, FLJ46903, UB1
	2.1	RAN binding protein 9	NM_005493	RANBPM
	2.1	ADP-ribosylation factor guanine nucleotide-exchange factor 1 (brefeldin A-inhibited)	NM_006421	ARFGEP1, BIG1, D730028O18Rik, P200

was used in this study to validate microarrays, and both participate in the regulation of endothelial activation.

S100A9 has been related to phosphorylation of cells in the arterial wall, NADPH-oxidase activity, and fatty acid transport, also being implied in arteritis of human giant cell, high levels of calcium-binding proteins [22].

Neither of these two proteins had been studied in PPHN, in spite of the fact that S100A9 has been

related to acute inflammatory diseases, especially in the lung, with an increase in proinflammatory cytokine levels such as interleukin 6, TNF alpha and C-reactive protein, and also plays an important role in cellular proliferation; that could explain in part the vasoreactivity and vascular remodeling in PPHN [23]. S100A12 has been suggested as a biomarker for spontaneous preterm birth [24] and a contributor in leukocyte migration in chronic inflammatory responses [25].



**Figure 3.** The upper panel shows the RSC1A1 mRNA expression of two representative samples corresponding to the control and persistent pulmonary hypertension of newborn (PPHN) groups. The lower panel represents a semi-quantitative analysis of RSC1A1 expression. Data represents a mean  $\pm$  standard deviation from 12 duplicated samples. Statistically significant respect to control at  $p < 0.05$ .

Among other biochemical alterations, matrix metalloproteinase expressions are increased in persistent vasoconstriction of smooth muscle, experimental pulmonary arterial hypertension, and in the remodeling of pulmonary arteries [26]. The up-regulation found of matrix metalloproteinase 9 suggests its participation in the degradation of the extracellular matrix.

### Hypoxia

Hypoxia and shear stress, recognized triggers in the development of vascular remodeling, alter cellular metabolic signaling pathways that regulate proliferation, migration and other components of pulmonary vascular diseases [27, 28].

Understanding the mechanisms responsible for regulating reactive oxygen species (ROS) production in various cell types, the targets of oxidant signals and ROS contributions to pulmonary vascular disease, is important for both pathogenesis and potential treatments. Circulating red blood cell can modulate nitric oxide (NO) signaling in the lung vasculature by viscosity and shear-mediated mechanic transduction of endothelial NO synthase activation, by hemoglobin-dependent NO scavenging, and by production of vasodilator mediators such as nitrite and ATP. Red cell hemolysis releases vasoconstrictive factors

such as hemoglobin and arginase-1 that produce endothelial dysfunction [29, 30].

The lack of surfactant protein D in PPHN contributes to pulmonary homeostasis [31]. Several conditions can trigger an increase in pulmonary vascular resistance and consequently PPHN. Our group has previously described the increased expression of HIF-1 alpha mRNA in neonates with PPHN [32]; however, the modulation of molecules involved around the over-expression of HIF-1 alpha has been seen in white blood cells and not vascular cells [33].

### Nucleotides metabolism and Rho

Of the more than 20 human proteins identified as part of Rho's family, Cdc42 and Rho GTPase, two of the three members most involved in vesicle trafficking, were studied. Cdc42 is a binding protein, highly involved in cytoskeletal control, which partially allows modifying the activity of actin and the regulation of cell polarity by a process of endocytosis [34]. The second member is part of the Rho GTPase family [35]. In this analysis, an up-regulation of 3 and 11A families were found.

Abnormal smooth muscle cell growth and function due to altered potassium and calcium channel expression and activity, increased calcium sensitization via enhanced Rho kinase activity, changes in mitochondrial or metabolic functions and other mechanisms are critical in PPHN. The NO-cyclic guanosine monophosphate (cGMP) system and prostacyclin-cyclic adenosine monophosphate (cAMP) system are particularly active in the pulmonary circulation of neonates. The biologic effects of NO are mediated by soluble guanylate cyclase in the vascular smooth muscle, which promotes the conversion of guanosine triphosphate (GTP) to cGMP, that, within the smooth muscle cell, activates specific protein kinases to decrease the  $Ca^{+2}$  influx into the cell and produce relaxation and vasodilation. In contrast, activation of signaling through the Rho GTPases and Rho kinase increases the  $Ca^{+2}$  sensitivity and stimulates the contractile apparatus [36, 37].

### Metabolism involved ionic transporters

Ionic transporters in acute alveolar hypoxia, through mechanisms such as the inhibition of  $K^{+}$  channels, membrane depolarization and calcium entry through voltage gates, and L-type  $Ca^{+2}$  channels, are responsible for pulmonary

smooth cells vasoconstriction, which redistributes desaturated and mixed venous blood away from poorly ventilated lung areas. Differences held in  $K^+$  and  $Ca^{+2}$  channels in response to hypoxia may help explain the hypoxic pulmonary vasoconstriction [38]. In this assay, several Rho GTPases were up-regulated. Rho is an enzyme which by phosphorylation of a myosin light chain increases muscular cell sensibility to calcium, producing a maintained vasoconstriction of smooth muscle. Rho kinase may be activated by hypoxia, increasing a sustained vascular resistance [39].

Rho kinase is a therapeutic target for the management of pulmonary hypertension in humans [39]; the presence of Rho kinase inhibitor in this study suggests its involvement in the pathophysiological mechanism of PVR. Nossaman et al. have suggested that Rho kinase has a critical role due to its capacity to promote vasodilatation independently of the mechanism that triggers the increase of  $Ca^{+2}$ , which leads to vasoconstriction and pulmonary hypertension [40].

Current treatment for PPHN is based on the blockage of the RhoA/Rho kinase via (ROCK) with Fasudil, which apparently mediates vasoconstriction sharing different signaling points in the pathogenesis of the disease [41].

The identification of markers involved in PPHN is essential for the understanding of the pathophysiology of the disease and the potassium channels play a role in regulating vascular smooth muscle function or vascular tone [42]. Microarray analysis made in this study exhibits the expression of potassium channels in PPHN by an up-regulation of voltage-gated  $K^+$  channel function and other potassium regulated channels of  $K^+$  subfamily. Mandegar and Yuan have proposed an alteration in the down expression of potassium channels leading to vasoconstriction [43]. The hypoxia inhibits the expression of  $K^+$  channels, affecting cell polarity, inducing an elevation of  $Ca^{+2}$  in cytosol and triggering vasoconstriction and vascular remodeling [42, 43].

Angiogenic factors, receptors, channels and transporters play a critical role in vascular signaling transduction being key elements in the pathogenesis of pulmonary vascular disease, each of them providing front line mechanisms for the regulation of normal smooth muscle. Furthermore, the expression of various molecules is affected by the pathologic remodeling of the PPHN. Improvement in understanding the systems which regulate these signals plays a pivotal role

in identifying the potential therapeutic approaches [44]. However, all the gene expression studies were done from blood (which would not represent the endothelial cells or lung tissues changes), which would be practically impossible to achieve in neonates, situation that represents a limitation, as well as the small number of patients. However, we are planning to enlarge the sample size in the next future.

## Conclusions

In the present study, the identification of the genes expressed at both the up- and down-regulation in neonates with PPHN explains in part the pathophysiology and structural changes characteristic of this serious disease. Based on our knowledge, this is the first study that explores the expression of these specific genes in PPHN. This may represent an important step to deep in the knowledge on pathophysiology and, consequently, possibly open new windows of therapeutic opportunities

## Declaration of interest

The Authors declare that there are no conflicts of interest regarding the publication of this paper.

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