Research Article

Searching for New Biomarkers in Pulmonary Hypertension Due to Left Heart Disease: Results from a Protein Array Analysis

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Abstract

Background: Pulmonary hypertension (PH) associates with high morbidity and mortality, being its main cause left heart diseases. Attending to its hemodynamic profile it can be divided in postcapillary or precapillary PH. The aim of this study was to search for new potentially useful biomarkers in this disease.

Methods: Patients who underwent a right heart catheterization were recruited and a blood sample from pulmonary trunk was obtained. Samples were divided in three groups of eight individuals, according to their hemodynamic profile (no PH, postcapillary PH, precapillary PH) and then mixed to obtain three pooled samples. The microarray Human L-series 1.000 from Ray Biotech was then performed.

Results: A thousand proteins were analysed. Among them, 67 showed significant expression differences. Seventeen proteins had a significant progressive increase (more that 1.5-fold) between two consecutive groups. These proteins were angiopoietin-like 4 (ANGPTL4), transient receptor potential cation channel subfamily C6 (TRCP6), transient receptor potential cation channel subfamily M7 (TRPM7), and opelin receptor (APJ); myocardial-related ones such as glycogen phosphorylase iso enzyme BB (GPBB), cathepsin B and osteocrin-musclin; ubiquitin; matrix metalloprotease 7 (MMP7); transcription factors as Kruppel-like factor 4 (KLF4), LIN41, RIP1 and TRAILR2; and immunity proteins as LIF, thymic stromal lymphopoietin (TSLP), LIMPII and interleukin 17B receptor.

Conclusions: Seventeen proteins, which correlate with the severity of PH due to left heart disease, have been identified. Further quantitative studies are needed to identify their potential use as biomarkers.

ABBREVIATIONS

PH: Pulmonary Hypertension; PAH: Pulmonary Arterial Hypertension; Mpad: Mean Pulmonary Arterial Pressure; LHD: Left Heart Disease; RHC: Right Heart Catheterization; NPH: Absence of PH; PPH: Postcapillary PH; CPH: Combined PH; TPG: Transpulmonary Gradient; ANGPTL4: Angiopoietin-Like Protein 4; TRCP6: Transient Receptor Potential Cation Channel Subfamily C6; TRPM7: Transient Receptor Potential Cation Channel Subfamily M7; APJ: Receptor; GPBB: Glycogen Phosphorylase Isoenzyme BB; MMP7: Matrix Metalloprotease 7; KLF4: Kruppel-Like Factor 4; TSLP: Thymic Stromal Lymphopoietin; ANGPT2: Angiopoietin 2; AXA7: Annexin A7; VEGF: Vascular Endothelial Growth Factor; EPCAM: Epithelial Cell Adhesion Molecule; FVw: Von Willebrand Factor; CHGA: Chromogranin A; TIMP2: Protein Tissue Inhibitor Of Metalloproteases 2; BMPR1A: Bone Morphogenetic Protein Receptor 1A; ROCK2: Rho-Kinase 2; GAL1: Galectin 1; TLR4: Toll Like Receptor 4; TSP4: Thrombospondin 4

INTRODUCTION

Pulmonary hypertension (PH) is a nosological entity defined by a raise of pulmonary vascular resistances (PVR) due to molecular and anatomical changes in the pulmonary circulation that lead to the development of right heart failure (HF) and, eventually, to death [1,2].

PH is defined as a hemodynamic condition in which the mean pulmonary arterial pressure (mPAP) is ≥ 25 mmHg at rest. PH can be classified in 5 groups according to the currently accepted classification of Dana Point [1,3,4]. Pulmonary wedge pressure (PWP) is an imperative hemodynamic measure, in order to identify the PH group and to derive other important parameters as PVR or cardiac output (CO).

The main cause of PH in our environment is left heart disease (LHD) (Dana Point group 2). Furthermore, PH is associated in patients with HF (both reduced and preserved ejection fraction) or valvular heart diseases [5,6]. When diagnosed, PH is associated with poorer outcomes [7,8]. The real prevalence of PH-LHD is not completely known (varies between 25 and 100% of the studied populations) because of the heterogeneity of the epidemiologic studies available in the literature regarding the population composition, echocardiographic cut-offs, etc [9-10]. Currently, echocardiography is the best available non-invasive diagnostic tool but involves severe limitations and biases [11]. For this reason, invasive tests as right heart catheterization (RHC) are needed, in order to achieve a proper diagnosis.

In PH, pulmonary arteries have developed structural and functional abnormalities, resulting in a loss of the endothelial regulation of the arterial tone (due to an imbalance between vasodilator and vasoconstrictor molecules) and in a activation of inflammatory and proliferative pathways [12,13]. We believe those abnormalities could be reflected in the blood flow by an altered expression molecules. The aim of this study was to search for novel and potentially useful non-invasive biomarkers related to the clinical and hemodynamic severity of this disease.

METHODS

Patient selection and classification according to the hemodynamic profile

An observational study in 24 consecutive patients diagnosed as left HF or other LHD who underwent a RHC was conducted at the Cardiology Department of the “Hospital Universitari i Politècnic La Fe”. Patients with evidence of PH due to other causes different from LHD (such as pulmonary diseases, pulmonary arterial hypertension (PAH) or pulmonary thromboembolism) were excluded from the study. The Ethics Committee for Clinical Research of the “Hospital Universitari i Politècnic La Fe” approved the study and informed consent was obtained from all participants in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki, and other institutional regulations.

RHC was performed at the catheterization laboratory of the “Hospital Universitari i Politècnic La Fe” following clinical indications and standardized protocols, selecting the right femoral vein as venous access. A 6F diagnostic Berman balloon-catheter (Arrow International Inc®, Reading, Pennsylvania, USA) was used to register the pressures of the different right heart chambers and the pulmonary artery. During the procedure several pressures were recorded: systolic, diastolic and mean pulmonary pressure (sPAP, dPAP, mPAP respectively), pulmonary-wedge pressure (PWP), systolic and diastolic right ventricle pressures and right atrium pressure. Additionally, a small blood sample from pulmonary artery was taken in order to perform a venous blood oximetry (SvO₂) (indispensable for further hemodynamic calculations). In case of an available arterial access, a sample of arterial blood was also taken and a blood oximetry was performed. In other cases, arterial blood saturation (SaO₂) was obtained from pulse oximeter. Body surface area (BSA) was calculated using patients’ weight and height; hence oxygen consumption (VO₂) was estimated.

Once provided all these hemodynamic parameters, cardiac output (CO) and PVR could be calculated, as well as other additional valuable parameters as transpulmonary pressure gradient (TPG) or diastolic pressure difference (DPD), applying the following formulae: TPG = (mPAP-PWP); DPD = (dPAP-PWP); CO (Fick) (l/min) = VO₂ / 1.3*Hemoglobin (g/dl) * (SaO₂ – SvO₂); Vo²(ml)= 135 ml O₂/min/M* BSA; BSA (Dubois) (m²) = [0.7148 x height (cm)⁰.⁷²⁵ x weight (kg)¹⁵⁴²⁵] / 100 and PVR (Wood units) = (mPAP – PWP) / CO.

All patients were classified within 3 different groups according to their hemodynamic profile: absence of PH(NPH), postcapillary PH (PPH), and combined PH (considered as transpulmonary pressure gradient TPG >= 12 mmHg) (CPH).

Processing of samples and protein profiling by antibody arrays

A 20 mL blood sample from pulmonary trunk was obtained from each patient (prior to wedging). Each sample was collected in dry tubes and the serum was respectively separated by centrifuging 1500 g 30 minutes at 4°C. Then, the serum specimen was aliquoted in Eppendorf cryotubes and stored at -80°C.

Preserved serum samples were selected and equal volumes were mixed to obtain a representative serum pool from each HP category. Then, pooled samples were processed in a Human L-Series Biotin Label-based Antibody Array 1000 system (Ray Biotech, Tebu-Bio, Le Perray-en-Yvelines, France). Fluorescence signal intensities of the glass chips were measured by using a laser scanner. This assay provides only a semi quantitative measurement of the included proteins. The background subtraction and normalization of the fluorescence intensity data to the internal and positive controls (printed onto each protein array chip) were considered before proceeding to the analysis. Next comparison of signal intensities, between and among array images from the different pools of samples, was used to determine relative differences in expression levels of each protein. Any ≥1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte was considered a measurable and significant difference in protein expression, provided that the raw signal intensity was above twice the maximum intensity of the negative control spots on the array (mean background ± 2 standard deviations, accuracy ≥ 95%, according to the manufacturer’s instructions).
In order to avoid spurious results, we only considered those proteins which presented significant and progressive increases in expression between consecutive groups (CPH>PHH>NPH) and those with the most remarkable differential expressions: more than 3-fold between NPH and CPH. Those proteins with just slight significant differences or with an abnormal expression progression between consecutive groups were ignored.

RESULTS

Patient characteristics

The main characteristics of the patients, classified according to its hemodynamic profile, are shown in (Table 1).

None of the clinical features showed significant differences among groups except those variables referring RHC hemodynamic measurements. However, as more severe was the PH, lower tended to be left ventricular ejection fraction (LVEF), poorer tended to be RV function, and larger tended to be RV diameter.

Protein profile of PH patient groups

Among the 1,000 proteins studied, 67(6.7%) showed significant expression differences. Among those 67, only 17 proteins presented progressive increases in expression between consecutive groups. Those proteins were endothelium-derived ones as angiopoietin-like protein 4 (ANGPTL4), transient receptor potential cation channel subfamily C6 (TRCP6), transient receptor potential cation channel subfamily M7 (TRPM7) and apelin receptor (APJ); myocardial-related ones such as glycogen phosphorylase isoenzyme BB (GPBB), cathepsin B and osteocrin-musclin; ubiquitin; matrix metalloprotease 7 (MMP7); transcription factors as Kruppel-like factor 4 (KLF4), LIN41, RIP1 and TRAILR2; and immunity proteins as LIF, thymic stromal lymphopoietin (TSLP), LIMPII and interleukin 17B receptor (Figure 1).

Other 50 proteins, although their expression showed a noteworthy progressive increase between NHP and CHP groups, did not reach significant differences between consecutive groups. Among those 50 proteins there are pathophysiologically remarkable ones as the endothelium-derived angiopoietin 2 (ANGPT2), annexin A7 (AXA7), vascular endothelial growth factor (VEGF), epithelial cell adhesion molecule (EPCAM) and Von Willebrand factor (vWF); heart markers as the fatty acid binding protein heart type (H-FABP) and chromogranin A (CHGA); coagulation by-products as fibrinogen or D-Dimers; vascular wall proteins as protein tissue inhibitor of metalloproteases 2 (TIMP2), bone morphogenetic protein receptor 1A (BMPR1A) and 80-kinase 2 (ROCK2); and others recently related to PH in animal models as galectin 1 (GAL1), FOXO1, toll like receptor 4 (TLR4), and thrombospondin 4 (TSP4) (Figure 2).

DISCUSSION

In this paper, we have assessed a total of 1,000 different proteins, belonging to different signalling pathways and biochemical routes, in serum samples from PH-LHD patients. Therefore, our study brings the possibility of exploring new potentially useful biomarkers that could lead to a better understanding and management of this disease.

The most outstanding proteins that showed remarkable expression differences between the PH-LHD patient groups were: KLF4, osteocrin-musclin, APJ, TRPM7, TRCP6, MMP7, GPBB, cathepsin B, ANGPTL-4, TSLP, LIN41, LIMPII, RIP1, TRAILR2, and others recently related to PH in animal models as galectin 1 (GAL1), FOXO1, toll like receptor 4 (TLR4), and thrombospondin 4 (TSP4).
Figure 1 Bar plot representing the intensity expression in fluorescence units of 15 proteins that achieved progressive significant differences.

Figure 2 Bar plot representing the intensity expression in fluorescence units of 20 proteins that achieved the most remarkable differences between patients without pulmonary hypertension and those with combined pulmonary hypertension.
LIF, and interleukin 17B receptor. All these proteins displayed significant and progressive increases in expression according to the higher severity of the hemodynamic characteristics among the patient groups. In addition, 50 proteins achieved remarkable expression differences between NPH (control patients) and CPH (sickest PH-LHD patients).

Among the 17 proteins with the best results on the microarray, only a few have been related with PH, either in animal or in vitro models. Apelin is an endothelium-derived hormone with a paracrine action. It can lead vascular smooth cells to apoptosis or inhibit its proliferation [14]. When infused to rats with monocrotaline-induced PH, an improvement in RV function was observed, suggesting it could be a potential new therapeutic target [15]. TRCP6 is a calcium channel predominately expressed in precapillary pulmonary arterioles. A marked contribution to hypoxia-induced PH has been reported [16], but this effect can be reversed with bosentan [17]. KLF4 is a zinc-finger transcription factor with a key role in cellular differentiation and proliferation. It is expressed in vascular smooth muscle cells and endothelial cells. It promotes anti-inflammatory and anticoagulant states, and increases endothelial nitric oxide synthase expression [18]. In fact, KLF4 knockout mice develop severe pulmonary hypertension in response to chronic hypoxia [19]. Ubiquitin should be discussed separately as it is one of the best-conserved proteins in the evolution. It participates of the intracellular protein degradation mechanism, labelling the proteins that are going to be destroyed. Disorders of this pathway have been related to the development of PH in humans [20] and rats [21].

Among the 17 proteins, there are several ones that take part of the homeostasis of the vascular bed, but a potential role in PH pathogenesis has not yet been assessed. ANGPTL4 is an angiogenic factor that is induced in endothelial cells as a response to hypoxia, protecting them from apoptosis [22]. In contrast, TRPM7 is a non-selective cation channel expressed in endothelial cells that contributes to the physiopathology of vascular systems by regulating cell adhesion and tube formation [23,24]. On the other hand, MMP7 or matrilysin has little paper known in cardiopulmonary physiology. It is known that MMP7 promotes smooth muscle apoptosis [25]. Besides, osteocin, also known as musclin, is a protein initially related to bone metabolism, but it has been recently classified within the family of mammalian natriuretic peptides [26]. Moreover, osteocin selectively binds to the NPR-C (natriuretic peptide clearance) receptor and modulates the response of cells to other natriuretic peptides, presumably increasing their availability [27]. Also cathepsin B, a lysosomal cysteine protease involved in degradation of the extracellular matrix, has never been studied in PH. Cathepsin B has been involved in apoptosis, degradation of myofibrillar proteins in myocardial infarction [28] and its inhibition reduces inflammatory damage in several animal models [29]. Additionally, GPBB is a glycogen phosphorylaseisoenzyme expressed in the cardiac muscle. Its levels rise with the myocardial damage [30]. Several studies have assessed its performance in early diagnosis of acute coronary syndromes [30], but without achieving relevant success [31]. Finally, LIMP-2, codified by the gene SCARB-2, is a protein expressed in the intercalated disks of the cardiomyocytes membranes. Abnormal levels have been associated with hypertrophic cardiomyopathy [32].

The remaining six proteins have no known relationship with PH or cardiovascular physiology. Three are inflammation related proteins (TSLP, LIF and L17Rlb) and the other three cell cycle regulatory proteins (LIN41, TRAILR2 and RIP1).

Among the other 50 proteins which achieved a noteworthy expression difference between NHP and CHP groups, there are several deserving to be remarked as they have been related to PHin previous studies. For instance, fibrinogen or D-dimers have been proposed as possible prognostic biomarkers in PH group 4 [33] and PAH [34] respectively. Also, H-FABP has been studied in group 4 PH and in heart failure patients, standing out as a potential survival predictor [35,36]. Further, angiotensin 2 is an independent survival predictor in PAH. Moreover, its levels correlated with mPAP and PVR [37]. Several studies have been performed with vWF. It is also an independent survival predictor in PAH [38], but also its levels are more elevated in patients with decompensated heart failure than in patients with stable heart failure and healthy controls [39]. Besides, TLR4, an innate immunity receptor has been associated with PH in animal models [40]. Additionally, ROCK2 is an enzyme expressed in arterial smooth cells. Its activity is enhanced in PAH patients [41] and, in a murine model of PH, PH severity decreased by inhibiting ROCK2 [42]. Also BMPR1A has been related with hereditary causes of PAH [43]. Its expression is reduced in the arteries of PH patients [44]. To conclude, VEGF is a proangiogenic factor with a supposed key role in PH pathogenesis. However, the underlying mechanisms have not been clearly determined [45]. On one hand, VEGF has demonstrated a protective effect mediated by a decrease of ET-1 production [46]. Murine models blocking VEGF receptor develop severe PH [47]. On the other, elevated levels of VEGF have been reported in patients with PAH [48] and in patients with PH and congenital heart diseases [49].

The current clinical practice guidelines recommend the use of brain natriuretic peptide (BNP) or its N-terminal fragment (NT-proBNP) as prognostic biomarkers in PH1. However, in PH-LHD, the increase of these peptides is inherent to heart disease; hence they are not useful in this condition. Currently, there is a complete lack of analytical parameters or surrogate biomarkers that could be used in PH-LHD. Therefore, the available literature shows several published articles where a substantial number of proteins have been assessed as potential biomarkers in PAH. Firstly, increased expression levels of endothelin 1 (ET-1) have been found in the lungs of a wide variety of patients with PH [50,51]. One study showed that ET-1 levels were raised and correlated with pulmonary pressures (mPAP and sPAP) and PVR in LHD patients with precapillary PH [52]. Relative to these studies, our results also showed the expression levels of ET-1 were higher in CPH group and, therefore, in group 4 PH and in heart failure patients, standing out as a potential survival predictor [35,36]. Further, angiopoietin 2 is a proangiogenic factor with a key role in PH pathogenesis. However, the underlying mechanisms have not been clearly determined [45]. On one hand, VEGF has demonstrated a protective effect mediated by a decrease of ET-1 production [46]. Murine models blocking VEGF receptor develop severe PH [47]. On the other, elevated levels of VEGF have been reported in patients with PAH [48] and in patients with PH and congenital heart diseases [49].

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showed interleukin 6 correlated with pulmonary pressures, regardless of ventricular function or HF etiology, and resulted as an independent predictor of sPAP. Even though dozens of immunity related proteins has been assessed in the protein array, only TSLP, a hemopoietic cytokine associated with immunity in various inflammatory diseases [61], achieved significant results. However, there is no known relationship between the TSLP protein and the PH or endothelial physiology. Regarding metalloproteases, MMP2 has been found at higher levels in PAH patients [62]. Our results showed higher, but not significantly different, expression levels of MMP2 in CPH group compared to NHP. In addition, MMP7 showed significant and progressive expression differences among the different groups of patients. Moreover, we detected the metalloprotease inhibitor TIMP-2 with lower expression in CPH compared with NHP. TIMP2 could play an interesting role in the regulation of endothelial remodeling, as in addition to inhibit metalloproteinases, it has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells in response to angiogenic factors [63].

Our study has several limitations that must not be ignored. First, we have chosen this commercial array chosen among others because it included most of the proteins we were interested to assess. However, there are other proteins of potential interest as nitric oxide synthase or natriuretic peptides that were not included. Second, we completed 1000 protein analysis; therefore the possibility of spurious results is substantial. Third, each study group is composed of only 8 individuals and their samples were pooled together. In this condition, isolated extreme values can affect the measurements, leading to wrong interpretations. Finally, as we only patients with clinical indication for RHC (those with a more severe condition), the sample is biased and the whole spectrum of the disease is not collected.

Despite all the limitations, our study is of interest as it is the first to study a broad collection of proteins in PH-LHD. This novel approach serves as starting point in the study of biomarkers with a more severe condition), the sample is biased and the whole spectrum of the disease is not collected.

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In conclusion, the present study has contributed to the screening of possible PH-LHD biomarkers by unveiling remarkable differences in the expression of several proteins in serum samples from patients. Therefore, these results deserve further specific quantitative studies in larger series of patients in order to validate their role in PH-LHD, their implication in non-invasive PH diagnosis and their true potential as possible therapeutic monitors.

REFERENCES


