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Pediatric Pulmonary Hypertension is Associated With Increased Circulating Levels of BMP 7 and CHIP

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ABSTRACT

Pulmonary arterial endothelial and smooth muscle cell homeostasis is regulated through the bone morphogenetic protein (BMP) and transforming growth factor beta (TGF-β) receptor pathways. Pathway imbalance results in pulmonary hypertension (PH). Each pathway has ligands and modulators influencing this balance. How these pathways differ in pediatric PH patients is unknown. Ten PH and 20 control subjects (ages 2–17 years) were prospectively enrolled. Pulmonary artery serum BMP 2, 4, 6, 7, 9, 10, activin A, TGF-β1, carboxyl terminus of Hsc70-interating protein (CHIP), NT Pro BNP, and CRP were measured by ELISA. Analyses were made using the Fisher's exact test, the Mann–Whitney test, ROC analysis, and Pearson and Spearman correlations as appropriate. PH subjects were group 1 (four with simple shunts) or group 3 PH. Control subjects had shunts scheduled for catheter closure but no PH. Only BMP 7 and CHIP levels were statistically elevated in PH patients versus controls; (BMP 7 0.081(0.076–0.084) vs. 0.074(0.069–0.08) OD, $p = 0.044$), (CHIP 0.17(0.14–0.24) vs. 0.13(0.12–0.15) OD, $p = 0.007$) respectively. BMP 7 levels correlated with RV systolic pressure (0.431, $p = 0.02$) and pulmonary resistance (0.446, $p = 0.013$). CHIP correlated with mean pulmonary artery pressure (0.449, $p = 0.013$) and resistance ratios (Rp/Rs) (0.419, $p = 0.02$). BMP 7 OD of 0.077 had sensitivity/specificity of 80% and 70% for PH. CHIP OD of 0.136 had sensitivity/specificity of 90% and 65% for PH. BMP 7 and CHIP levels are heightened in pediatric PH patients which correlate with catheterization values. BMP 7 and CHIP could provide sensitive markers for PH to aid in diagnosis and disease monitoring.

1 | Introduction

Pulmonary hypertension (PH) affecting children continues to remain a life-threatening condition with a prevalence of roughly 18 per one million children [1]. PH is characterized by pulmonary artery endothelial cell dysfunction and excessive proliferation of pulmonary artery smooth muscle cells, leading to abnormal vasoconstriction and pulmonary vascular remodeling [2]. These changes result in the escalation of pulmonary artery pressure causing right heart strain, which can rapidly progress to right heart failure and eventually death [3, 4]. Until the recent approval of sotatercept, therapies for PH targeted mainly vasoconstriction through stimulation of nitric oxide pathway, inhibition of endothelin pathway, and activation of prostacyclin pathway [5, 6]. Though progress has been made with these treatment advancements, the 1 and 10-year survival in pediatric PH patients is about 87% and 69%, respectively [1].

The remodeling of pulmonary circulation in PH has been linked to the dysregulation of the transforming growth factor-β (TGF-β) superfamily, which consists of two interacting pathways; the TGF-β-activin-nodal pathway and the bone morphogenetic protein (BMP) growth and differentiation factors (GDF)

pathway [3, 4, 6]. Each pathway has specific ligands that interacts with unique cell membrane receptors that in turn activates a specific signal transduction cascade, and to gene transcription [6]. These pathways must remain in balance in order to have stable cellular growth and function [6]. BMP 7 and carboxyl terminus of Hsc70-interating protein (CHIP) serve important roles as negative feedback regulators of these pathways, thereby promoting balance in cellular function [7, 8].

This pilot study evaluated the levels of known ligands and protein modifiers that affect the BMP and TGF-β pathways in a group of stable pediatric PH patients and control subjects. The purpose of this study was to identify significant ligands/modifiers that might serve as biomarkers for the presence of PH and correlate with hemodynamic data obtained by cardiac catheterization.

2 | Methods

2.1 | Study Participants and Subject Selection

This was a single-center prospective study performed at Children's Wisconsin. The study was approved by the Institution regulatory body at Children's Wisconsin. All participants provided written permission by informed consent/assent and the study was approved by the Children's Wisconsin Internal Review Board (PRO 45920). The study was conducted in accordance with Helsinki Declaration as revised in 2013 and no authors had any conflict of interest.

All patients were between 2 and 17 years old at the time of enrollment. PH patients were followed clinically at the Herma Heart Institute pulmonary hypertension clinic and had either group I (pulmonary arterial hypertension with or without associated congenital heart disease [CHD]) or group 3 (PH due to developmental lung disease) PH based on the 6th World Symposium on Pulmonary Hypertension (WSPH) criteria [9] at the time of study sample collection and were clinically stable on pulmonary vasodilator therapy. PH patients were all clinically stable and were scheduled for routine right heart catheterization and had mean pulmonary artery pressures > 20 mmHg, pulmonary vascular resistance index (PVRI) values > 3 Woods units $\times$ m^2, and capillary wedge pressures < 15 mmHg at catheterization [9]. Catheterizations were done under general anesthesia for children < 12 years old, children with anxiety issues, and children undergoing intervention.

Control subjects were recruited when scheduled for an elective cardiac catheterization for either ASD or PDA device closure, and included if right heart catheterization confirmed they did not have PH. Patients with complex CHD, organ transplantation and on antirejection drugs or significant genetic abnormalities such as trisomies, large gene duplications, or deletions were excluded.

Study blood samples were obtained from the pulmonary artery of each subject for analysis. Clinical and imaging data were collected on each patient, which included World Health Organization (WHO) functional status, PH medications used and their duration, associated cardiac lesions and associated medical conditions.

2.2 | Measurements of Serum Ligand/Modifier Concentrations

The circulating levels of BMP 2, 4, 6, 7, 9, and 10 along with activin A, TGF-β1 and CHIP were measured using commercially available streptavidin ELISA assays (RayBiotech for all ligands except Booster for BMP 10 only). These were performed by a two-step sandwich assay with streptavidin-coated microtiter plates. The ELISA assay utilizes 50 μL plasma and were performed in duplicates. The lower detection limit for each protein using this assay varies per protein Elisa kit. We used the standard supplied with each kit to calculate the unknown in each sample. Results were reported by the raw optical density (OD) value. N-Terminal Pro-B-Type Natriuretic Peptide (NT Pro BNP) and CRP levels were measured through our hospital's standard laboratory.

2.3 | Statistical Analysis

The Fisher's exact test was used to compare categorical variables and are reported as n (%). The Mann–Whitney test was used to compare continuous variables and are reported as median (interquartile range [IQR]). Spearman correlations were used to compare study proteins and the presence or absence of PH. A p value < 0.05 was considered significant. A ROC analysis was performed for BMP 7 and CHIP OD levels. Thresholds were identified by maximizing Youden's Index. SPSS version 28 was used for analyses.

3 | Results

3.1 | Patient Characteristics

Thirty children were enrolled: 10 with PH and 20 with controls (Table 1). All PH patients were medically stable on background pulmonary vasodilator therapy with 9/10 having WHO functional class I or II and normal NT Pro BNP levels. Patients had PH diagnosis for a median of 8.1 years (2–16.8 years). Four group 1 PH patients had VSD's that were surgically repaired, and one group 3 PH patient had an associated restrictive ASD. None of the control patients were symptomatic from their shunt lesions. No patients were actively infected as shown by having normal CRP values (all 0). There were significant differences in catheterization pressures between the two groups as expected (Table 2).

3.2 | Concentrations of Study Target Markers and PH Status

Circulating levels of study proteins were measured and correlated with the presence or absence of PH by catheterization (Table 3). Of all the study proteins, only BMP 7 and CHIP were significantly elevated in the presence of PH [BMP 7 0.081 (0.076–0.084) vs. 0.074 (0.069–0.080) $p = 0.044$ and CHIP 0.17 (0.014–0.24) vs. 0.13 (0.12–0.15) $p = 0.007$ in PH vs. non-PH, respectively]. Using this data gave BMP 7 OD of 0.077 with sensitivity/specificity of 80% and 70% respectively for PH, while

TABLE 1 | Demographics.

	Non-PH ($n = 20$) n (%) or median (IQR)	PH ($n = 10$) n (%) or median (IQR)	p Value
Age, years	5.9 (3.7–8.8)	12.2 (7.4–15.4)	0.035
Female	11 (55)	8 (80)	0.25
PH group			NA
1	–	7 (70)	
3	–	3 (30)	
WHO Functional Class			< 0.001
I	20 (100)	1 (17)	
II	0 (0)	4 (66)	
II–III	0 (0)	1 (17)	
Associated shunts			0.0002
ASD	9 (45)	1 (10)	
PDA	11 (55)	0 (0)	
VSD	0 (0)	4 (40)	
PH medication type[a]			NA
PDEi	–	10 (100)	
ERA	–	8 (80)	
OG-O/I	–	5 (50)	
PG-P	–	2 (20)	

Abbreviations: ASD, atrial septal defect; ERA, endothelin receptor antagonist; IQR, interquartile range; PDA, patent ductus arteriosus; PDE5i, phosphodiesterase 5 inhibitor; PG-O/I, prostacyclin-oral/Inhaled; PG-P, prostacyclin-parenteral; VSD, ventricular septal defect.
[a]May have more than one medication.

TABLE 2 | Catheterization data average pressures (median and IQR).

	Non-PH subjects $n = 20$	PH subjects $n = 10$	p Value
RA	5.0 (4.3–7.0)	7.5 (5.0–9.0)	0.039
mPAP	14.0 (11.0–15.0)	32.5 (24.8–46.0)	< 0.001
PCWP	8.5 (7.0–10.0)	10.5 (8.5–12.0)	0.061
PVRI	0.85 (0.6–1.4)	8.0 (5.3–12.0)	< 0.001

Abbreviations: mPAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; PVRI, pulmonary vascular resistance index; RA, right atrium.

CHIP OD of 0.136 showed a sensitivity/specificity of 90% and 65% respectively for PH (Figure 1). This was a positive predictive value (PPV)/negative predictive value (NPV) of 57.1% and 87.5% for BMP 7 and 53.3% and 86.7% for CHIP, respectively.

3.3 | Correlations of BMP 7 and CHIP With Catheterization Data

Focusing on BMP 7 and CHIP, both had a significant moderate level of correlation with mean PA pressure when evaluating all patients (Table 4). BMP 7 also had a moderate level of correlation with RV systolic pressure and PVRI while CHIP had a moderate level of correlation with Rp/Rs ratio. For comparison NT Pro BNP, which is routinely used to monitor PH patients for myocardial dysfunction and risk assessment for therapeutic choices [10], did not show any significant correlation with these measurements.

4 | Discussion

This study which evaluated the blood levels of key proteins involved in the TGF-β superfamily found that only circulating BMP 7 and CHIP were significantly elevated in pediatric PH patients compared with controls. BMP 7 and CHIP also had moderate positive correlation with key cardiac catheterization pressure variables compared with the lack of correlation with NT Pro BNP. There was a good sensitivity of BMP 7 and strong sensitivity of CHIP which provided a very good NPV in assessing the presence of PH. The finding of elevated circulating BMP 7 and CHIP are significant and likely reflects the roles these proteins may have in maintaining balance between the TGF-β-activin-nodal pathway and the BMP-GDF pathway [3, 4, 6].

The TGF-β-activin-nodal pathway and the BMP-GDF pathway regulate cellular homeostasis, cell proliferation, migration, adhesion, differentiation, and apoptosis [3]. Each pathway has unique cellular receptors (composed of serine/threonine receptor kinases of type I and type II subtypes) that are activated by particular ligands (e.g., TGF-β subtypes and activins for the TGF-β-activin-nodal pathway and BMPs and many

TABLE 3 | Outcomes by ELISA testing.

ELISA test	Non-PH $n = 20$ Median (IQR)	PH $n = 10$ Median (IQR)	p Value
NT pro BNP level	93.0 (66.5–132.3)	128.5 (62.8–168.5)	0.502
BMP 2 avg OD	0.33 (0.30–0.41)	0.25 (0.16–0.47)	0.502
BMP 4 avg OD	0.18 (0.17–0.21)	0.21 (0.17–0.29)	0.131
BMP 6 avg OD	0.24 (0.13–0.38)	0.33 (0.20–0.68)	0.248
BMP 7 avg OD	0.074 (0.069–0.080)	0.081 (0.076–0.084)	0.044
BMP 9 avg OD	0.20 (0.17–0.44)	0.39 (0.18–0.63)	0.397
BMP 10 avg OD	0.28 (0.25–0.39)	0.29 (0.25–0.37)	0.746
Activin A avg OD	0.79 (0.56–1.05)	0.75 (0.38–1.42)	0.914
TGF-β avg OD	1.39 (1.13–1.84)	1.56 (1.04–2.44)	0.619
CHIP avg OD	0.13 (0.12–0.15)	0.17 (0.14–0.24)	0.007
CRP level	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.846

Abbreviation: OD, ocular density.

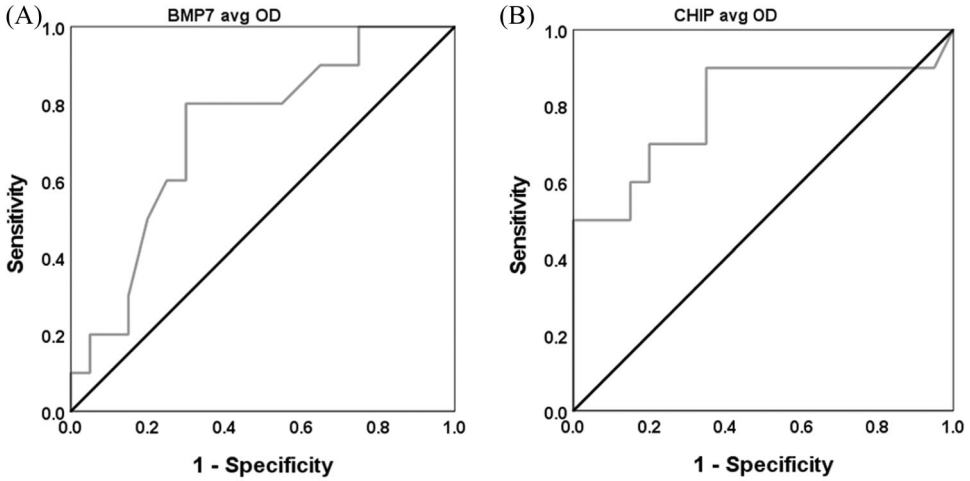

FIGURE 1 | (A) BMP 7 average Ocular density (OD): Area under the curve was 0.728, 95% CI (0.538–0.917) Cut-off: Sensitivity 0.8 and Specificity 0.7. (B) CHIP average OD: Area under the curve was 0.798, 95% CI (0.602–0.993) Cut-off: Sensitivity 0.9 and Specificity 0.65.

TABLE 4 | Catheterization data correlation (Spearman) with BMP 7, CHIP levels, and NT Pro BNP levels for all patients.

Catheterization value	BMP 7 average OD	CHIP average OD	NT Pro BNP level
RV systolic pressure	0.431, $p = 0.02$	0.304, $p = 0.108$	−0.047, $p = 0.810$
mPAP	0.359, $p = 0.051$	0.449, $p = 0.013$	−0.056, $p = 0.770$
PVRI	0.446, $p = 0.013$	0.338, $p = 0.068$	−0.011, $p = 0.955$
Rp/Rs	0.350, $p = 0.058$	0.419, $p = 0.021$	0.008, $p = 0.965$

Abbreviations: Rp/Rs, pulmonary resistance over systemic resistance; RV, right ventricle.

growth and differentiation factors for BMP-GDF pathway) [3, 4, 6, 7, 11]. Once a ligand activates the receptors, an intracellular cascade occurs by a series of SMAD proteins (SMAD 1/5/8 for BMP-GDF pathway and SMADs 2/3 for TGF-β-activin-nodal pathway) [4, 7, 12]. The end result is activation of SMAD 4, which then is able to translocate into the nucleus to increase target gene expression based upon the particular pathway [4]. These pathways work to keep each other in balance as they each appear to have opposite effects on gene targets and defects in any part of the pathway leads to imbalance [6]. When the imbalance promotes the TGF-β pathway, PAH can develop as a result of increased disease modulators such as inflammation, cell migration, differentiation, endothelial cell dysfunction, and aberrant angiogenesis in addition to several other downstream effects [4, 6]. Genetic mutations have been discovered that affect these pathways and have been linked to the development of PAH [4]. One of the first noted mutations was in the BMP receptor gene BMPR2 resulting in pathway inhibition and is

linked to 70%–80% of patients with inherited PAH and around 20% of patients with sporadic mutations [13].

BMP 7 along with BMP 2 and 4, is one of the most significant ligands involved in BMPR2 signaling, especially in pulmonary arteries [14]. BMP 7 is separate from BMP 2 and 4 in that is has more affinity for type II receptors, similar to how activin ligands do [14]. BMP 7 has been demonstrated in pulmonary artery endothelium and smooth muscle and is likely released by autocrine or paracrine means into the surrounding area [14]. After binding to BMP receptors, BMP 7 will activate the SMAD cascade 1/5/8 but also SMAD 7 that antagonizes TGF-β pathway [7]. Its role appears to counteract the functions of TGF-β [7]. Such effects include inhibition of fibroblast differentiation into myofibroblasts seen in vessel remodeling as well as inhibition of angiogenesis and inflammation [7, 15]. A study by Liu et al. demonstrated the potential for circulating BMP 7 measurement in adults with PAH [14]. They found that compared with control subjects, PAH patients had significantly higher measured blood levels of BMP 7 and in particular, patients with BMPR2 mutations had the highest BMP 7 levels compared other PAH patients [14]. Interestingly, our one patient with suspected BMPR2 mutation did have the highest BMP 7 level of our PH group (0.089). The Liu study also showed that BMP 7 levels were positively correlated with increased mortality in PH patients with "high" levels having a 5-year survival of only 42.2% versus 63.4% in the "low" level group [14]. Additionally, the Liu study did not show any correlation between BMP 7 levels and hemodynamic data. In contrast, we did find a moderate level of correlation in our study [14].

CHIP, also known as Stub1 is a ubiquitin ligase known as a U-box-dependent E3 ligase and a co-chaperon protein [16]. The ubiquitin ligases can offset the TGF-β/BMP pathway balance and increase cellular responsiveness to TGF-β family ligands by targeting Smad proteins for degradation in the proteasome [17–19]. This effect for the most part reduces the TGF-β signaling activity. For example, several E3 ligases, such as SCF/Roc1 and Smurf1/2, are thought to mediate the ubiquitination and degradation of the activated form of Smad proteins [20–26]. CHIP, originally identified as a tetra-trico-peptide repeat-containing protein, was confirmed to interact with heat shock proteins (Hsp) [27]. CHIP has been reported to inhibit the Hsp40-stimulated ATPase activity of heat shock cognate protein (Hsc) 70 and inhibit the Hsc70-substrate-binding cycle to reduce chaperon efficiency or enhance Hsp70-dependent folding activity [27, 28]. CHIP levels are increased during cellular stress, such as oxidation, which further induces the cochaperone Hsp70 [29]. Hsp70 can form a complex with CHIP and SMAD 3 that will inhibit SMAD 3 function and inactivate the TGF-β pathway [8]. This process is antagonized by Hsp90 [29]. It is therefore conceivable that CHIP has a cardioprotective function in PH and its protective function, is in part mediated by controlling the sensitivity of the TGF-β signaling by reducing the basal level of Smad3 via ubiquitination. Previously, CHIP has also been shown to interact with Smad1/4 and regulate the BMP-GDF pathway through SMAD degradation [16]. The overall net effect appears that CHIP serves to protect the cell from stress-induced apoptosis through enhancing degradation of misfolded proteins [29]. Ours is the only prospective clinical study we could find on circulating CHIP levels being elevated in the presence of PH compared with controls.

We noted that the other ligands, including activin A and TGF-β1 were not significantly elevated in pediatric PH patients compared with controls in this study. This is contrary to studies that have found both activin A and TGF-β1 are elevated in adult PAH patients compared with control subjects and positively correlate with mortality [14, 15]. Ligands such as activin A are now clinically targeted with new activin receptor type IIA-Fc fusion protein medications, such as recently FDA approved sotatercept. This is a novel PAH medication that affects the TGF-β pathway by ligand trapping molecules such as activin A [30]. Sotatercept was found in phase II studies to lower activin A levels in adult subjects with significant improvement in multiple clinical and hemodynamic parameters [30]. One potential reason for our finding of nonsignificant difference in activin A levels compared with controls could be explained in a mouse PAH model by Yndestad et al. [31]. They showed that activin A is elevated initially in experimentally induced PAH, however, these levels returned to control levels in a couple of weeks despite mRNA levels for the activin receptor II being elevated [31]. The authors felt this may imply adaptive down-regulation taking place from chronic PAH [31]. The effects of lowering activin A levels in pediatric PH is unknown but is currently under investigation [32].

4.1 | Limitations

This study is limited by the low number of PH patients due to being a single-center population. The PH subjects were a mix of group 1 (40% with CHD) and group 3 PH patients, which prohibits data from applying to one particular PH subgroup. Due to ethical issues, control subjects all had asymptomatic simple CHD that was scheduled for shunt closure. This may limit comparisons to children with structurally normal hearts. All but one of the PH subjects had normal NT Pro BNP level highlighting well-controlled PH, but it is unclear if these findings apply to decompensated patients. In addition, no follow-up lab testing was performed to follow trends over time or effects of medication changes.

4.2 | Interpretation

Circulating BMP 7 and CHIP levels were significantly elevated and had good hemodynamic correlations with cardiac catheterization measurements in clinically stable mixed group of PH patients compared with asymptomatic CHD controls. No correlation was found in other ligands affecting the TGF-β-activin-nodal/BMP-GDF pathways in this study sample. Therefore, BMP 7 and CHIP levels could provide sensitive markers for PH to aid in diagnosis and disease monitoring.

Author Contributions

Edward C. Kirkpatrick, Adeleye J. Afolayan, and G. Ganesh Konduri contributed to project design and laboratory methods. Edward C. Kirkpatrick, Adeleye J. Afolayan, G. Ganesh Konduri, and Todd M. Gudausky contributed to data collection. All authors contributed substantially to data analysis, manuscript writing and interpretation.

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Ethics Statement

This study is an original research article that was approved by the Institution regulatory body at the Medical College of Wisconsin.

Consent

All participants provided written permission by informed consent/assent and the study was approved by the Medical College of Wisconsin internal review board (PRO 45920).

Conflicts of Interest

The authors declare no conflicts of interest.

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