university of groningen

University Medical Center Groningen

University of Groningen

Early angiogenic proteins associated with high risk for bronchopulmonary dysplasia and pulmonary hypertension in preterm infants

Arjaans, Sanne; Wagner, Brandie D.; Mourani, Peter M.; Mandell, Erica W.; Poindexter, Brenda B.; Berger, Rolf M. F.; Abman, Steven H.

Published in:
American Journal of Physiology - Lung Cellular and Molecular Physiology

DOI:
10.1152/ajplung.00131.2019

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Arjaans, S., Wagner, B. D., Mourani, P. M., Mandell, E. W., Poindexter, B. B., Berger, R. M. F., & Abman, S. H. (2020). Early angiogenic proteins associated with high risk for bronchopulmonary dysplasia and pulmonary hypertension in preterm infants. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *318*(4), L644-L654. https://doi.org/10.1152/ajplung.00131.2019

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Am J Physiol Lung Cell Mol Physiol 318: L644–L654, 2020.
First published January 22, 2020; doi:10.1152/ajplung.00131.2019.

RESEARCH ARTICLE

Early angiogenic proteins associated with high risk for bronchopulmonary dysplasia and pulmonary hypertension in preterm infants

Sanne Arjaans,[1]* Brandie D. Wagner,[2,3]* Peter M. Mourani,[2]* Erica W. Mandell,[2] Brenda B. Poindexter,[4,5] Rolf M. F. Berger,[1] and Steven H. Abman[2]

[1]*Department of Paediatric Cardiology, Centre for Congenital Heart Diseases, Beatrix Children's Hospital, University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands;* [2]*Pediatric Heart Lung Center, Pediatrics, University of Colorado Denver, Aurora, Colorado;* [3]*Department of Biostatistics and Informatics, Colorado School of Public Health, University of Colorado Denver, Aurora, Colorado;* [4]*Section of Neonatal-Perinatal Medicine, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana; and* [5]*Perinatal Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio*

Submitted 15 March 2019; accepted in final form 15 January 2020

Arjaans S, Wagner BD, Mourani PM, Mandell EW, Poindexter BB, Berger RMF, Abman SH. Early angiogenic proteins associated with high risk for bronchopulmonary dysplasia and pulmonary hypertension in preterm infants. *Am J Physiol Lung Cell Mol Physiol* 318: L644–L654, 2020. First published January 22, 2020; doi:10.1152/ajplung.00131.2019.—Early pulmonary vascular disease in preterm infants is associated with the subsequent development of bronchopulmonary dysplasia (BPD) and pulmonary hypertension (PH); however, mechanisms that contribute to or identify infants with increased susceptibility for BPD and/or PH are incompletely understood. Therefore, we tested if changes in circulating angiogenic peptides during the first week of life are associated with the later development of BPD and/or PH. We further sought to determine alternate peptides and related signaling pathways with the risk for BPD or PH. We prospectively enrolled infants with gestational age <34 wk and collected blood samples during their first week of life. BPD and PH were assessed at 36 wk postmenstrual age. Samples were assayed for each of the 1,121 peptides included in the SOMAscan scan technology, with subsequent pathway analysis. Of 102 infants in the study, 82 had BPD, and 13 had PH. Multiple angiogenic proteins (PF-4, VEGF121, ANG-1, bone morphogenetic protein 10 [BMP10], hepatocyte growth factor (HGF), ANG-2) were associated with the subsequent diagnosis of BPD; and FGF-19, PF-4, connective tissue activating peptide (CTAP)-III, and PDGF-AA levels were associated with BPD severity. Early increases in BMP10 was strongly associated with the late risk for BPD and PH. We found that early alterations of circulating angiogenic peptides and others were associated with the subsequent development of BPD. We further identified peptides that were associated with BPD severity and BPD-associated PH, including BMP10. We speculate that proteomic biomarkers during the first week of life may identify infants at risk for BPD and/or PH to enhance care and research.

angiogenesis; aptamers; biomarkers; bronchopulmonary dysplasia; lung development; proteomics; pulmonary hypertension; SOMAmer

INTRODUCTION

Advances in perinatal medicine have increased survival of extremely premature infants over the past decades (47). However, preterm infants remain at high risk for late respiratory morbidity and mortality caused by the development of bronchopulmonary dysplasia (BPD), the chronic lung disease of prematurity that occurs in infants who have required respiratory support and oxygen therapy at birth (19, 38). BPD is a multifactorial disease that is associated with complex interactions between genetic, molecular, cellular, and environmental factors, but its pathogenesis is incompletely understood, and strategies to prevent BPD remain limited (2, 4, 16, 29). Preterm birth and postnatal injury to the developing lung can impair angiogenesis and alveolarization, resulting in simplification of the distal lung airspace. These characteristic histologic changes of BPD are clinically manifested by persistent respiratory disease with a prolonged need for supplemental oxygen, recurrent respiratory exacerbations with frequent hospitalizations (45), exercise intolerance, pulmonary hypertension (PH), and related respiratory impairments that can extend into adulthood (4).

PH often complicates the postnatal course and outcomes of extremely premature infants, especially in infants with severe BPD (7, 23, 35). Past studies suggest that 15%–25% of infants with BPD also develop PH, with the highest rates of PH reported in severe BPD (3, 7, 32, 35). When present, PH is associated with poor outcomes in BPD infants, with mortality rates of up to 48% (3, 22). A recent study suggests that the presence of PH by echocardiography within the first 2 wk of life is associated with poor in-hospital survival (6). Therefore, insights into factors that contribute to the pathogenesis of BPD and PH are needed to develop better strategies for the early identification and treatment of at-risk premature infants (29).

In addition to the important contribution of PH to the pathophysiology and cardiorespiratory outcomes of infants with BPD, abnormal growth of the lung circulation, or angiogenesis, may contribute to disease pathogenesis, including disruption of alveolarization (1, 18, 46, 49). Past studies have suggested that early disruption of pulmonary vascular growth, endothelial dysfunction, and impaired angiocrine signaling may contribute to the development of BPD (1, 8, 18, 44, 46, 54). Experimentally, disruption of angiogenesis in neonatal

* S. Arjaans, B. D. Wagner, and P. M. Mourani contributed equally to this work.

Address for correspondence: S. Arjaans, Dept. of Paediatric Cardiology, Beatrix Children's Hospital, University Medical Centre Groningen, Hanzeplein 1, P.O. Box 30.001, 9700 RB Groningen, Groningen, The Netherlands (e-mail: s.arjaans@umcg.nl).

1040-0605/20 Copyright © 2020 the American Physiological Society

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

animals can impair alveolar growth and cause PH, suggesting that early injury to the developing vasculature may contribute to sustained abnormalities of lung structure and function (18), which is referred to as the "vascular hypothesis" of BPD (1, 46, 49). Clinically, the presence of pulmonary vascular disease by echocardiogram at 7 days of age is associated with the subsequent development of BPD and PH at 36 wk postmenstrual age (PMA) (35) as well as late respiratory disease during early childhood (36). These findings suggest that early alterations in angiogenic signaling or related processes may be contributing to or reflect high risk of the subsequent diagnosis of BPD with or without PH at 36 wk PMA.

Past studies have suggested that proteomic biomarkers may be useful to identify infants with a greater likelihood of developing BPD (25). Markers such as inflammatory proteins [including increased interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and interferon-γ and decreased IL-10, IL-17, RANTES, and TNF-β)] and others have been associated with high risk for BPD (16, 30). In addition, changes in biomarkers in the cord blood, including such angiogenic proteins as VEGF, placental growth factor (PLGF), and sFlt-1, and early increases in brain natriuretic peptide (BNP) have been associated with a greater risk of developing PH in preterm infants, especially in the setting of abnormal placental vascular structure and intrauterine growth restriction (24, 30, 33, 48). However, whether early changes in circulating angiogenic proteins or other signaling pathways can provide strong predictors of risk for BPD and for PH in preterm infants is uncertain.

An aptamer-based proteomic platform has proven useful in identifying peptides that are strongly associated with disease phenotypes and course in diverse clinical settings (14, 27). Aptamer-based proteomic strategies have the ability to identify and accurately measure large numbers of peptides that often circulate at low concentrations and to reflect multiple signaling pathways from very small amounts of blood (41). This capability may be especially important in studies involving extremely preterm infants, as the use of large-scale assays has been partly limited in the past by the relatively large amounts of blood required. Forster et al. (12) recently applied this aptamer-based platform to associate circulating peptides with BPD risk; however, samples were obtained at different postnatal ages during the first month of life and included only a small case series of preterm infants.

Therefore, our objectives of this study were to first determine whether circulating angiogenic peptides as determined from a large-scale proteomic strategy during the first days after birth are associated with the subsequent diagnosis of BPD and its severity in preterm infants or are associated with the development of PH at 36 wk PMA. We further sought to identify other peptides and signaling pathways in addition to the angiogenic peptides that may be associated with BPD risk, BPD severity, and PH at 36 wk PMA.

METHODS

Study design and population. This study was supported by a National Institutes of Health (NIH)-funded, prospective observational study (RR-02192 to P. M. Mourani, PI, and HL-085703 to S. H. Abman, PI). The study protocol was approved by the Institutional Review Board, and written informed consent was received from the parents or guardians of all participants. We included infants with a gestational age of less than 34 wk at birth and a birthweight between 500 g and 1,250 g. Exclusion criteria included clinical evidence of congenital heart disease (except patent ductus arteriosus, patent foramen ovale, or hemodynamically insignificant atrial/ventricular septal defects), lethal congenital abnormality, and futile cases. Infants who died before the diagnosis of BPD could be made at 36 wk PMA were excluded. Subjects were enrolled between July 2006 and March 2013. Data was managed using a REDcap (Research Electronic Data Capture) (15) database hosted at the University of Colorado Denver as previously described (35).

Primary outcome. BPD status and severity were assessed at 36 wk PMA using a modification of the standard NIH workshop definition

Table 1. *Patient demographics*

Patient Characteristic	All (n = 102)	No BPD (n = 20)	Mild BPD (n = 34)	Moderate BPD (n = 26)	Severe BPD (n = 22)	P Value*	P Value†
Birth weight, g	856.5 (187.1)	1,083.0 (105.3)	832.3 (148.7)	803.2 (176.7)	751.1 (146.0)	<0.01	<0.01
Birth weight z-score	−0.29 (0.72)	−0.71 (0.75)	−0.15 (0.73)	−0.29 (0.66)	−0.15 (0.66)	0.03	0.02
Gestational age, wk	26.48 (2.07)	29.10 (1.52)	26.09 (1.40)	26.00 (1.85)	25.27 (1.67)	<0.01	<0.01
Maternal age, yr	27.28 (6.02)	29.50 (6.14)	26.38 (6.10)	26.35 (6.57)	27.77 (4.81)	0.24	0.32
Sex (male)	49 (48%)	12 (60%)	15 (44%)	10 (38%)	12 (55%)	0.44	0.96
Maternal race (white)	92 (90%)	18 (90%)	31 (91%)	23 (88%)	20 (91%)	0.99	0.99
Maternal ethnicity, Hispanic or Latino	30 (29%)	2 (10%)	10 (29%)	10 (38%)	8 (36%)	0.16	0.07
Multiple gestation	24 (24%)	8 (40%)	5 (15%)	6 (23%)	5 (23%)	0.21	0.38
Antenatal corticosteroids‡	78 (76%); n = 95	16 (80%); n = 18	24 (71%); n = 32	20 (77%); n = 25	18 (82%); n = 20	0.75	0.99
Cesarean section	73 (72%)	14 (70%)	24 (71%)	19 (73%)	16 (73%)	0.99	0.99
Intubated	93 (92%)	12 (65%)	33 (97%)	26 (100%)	22 (100%)	<0.01	<0.01
Surfactant	87 (85%)	9 (45%)	31 (91%)	25 (96%)	22 (100%)	<0.01	<0.01
Respiratory status at day 7							
Mechanical ventilation	52 (51%)	3 (15%)	18 (53%)	16 (62%)	15 (68%)	<0.01	<0.01
FI_{O_2}	0.30 (0.10)	0.26 (0.08)	0.29 (0.07)	0.31 (0.10)	0.35 (0.13)	0.04	0.26
Early PH‡	44 (43%)	6 (30%)	17 (51%); n = 33	10 (39%)	11 (50%); n = 21	0.35	
PH at 36 wk PMA‡	13 (13%)	0 (0%)	4 (12%); n = 33	3 (12%)	6 (27%); n = 21	0.08	0.04
PDA (medical treatment)	63 (62%)	3 (15%)	22 (65%)	19 (73%)	19 (86%)	<0.01	<0.01
PDA (surgical ligation)	24 (24%)	0 (0%)	5 (15%)	8 (31%)	11 (50%)	<0.01	<0.01

Values are means ± SD or n (%). All patient characteristics across bronchopulmonary dysplasia (BPD) groups are shown. FI_{O_2}, fraction of inspired oxygen; PDA, patent ductus arteriosus; PH, pulmonary hypertension; PMA, postmenstrual age. *P values displayed from comparison between all the severity groups of BPD; †P values displayed from comparison between severe BPD versus no BPD. ‡Second n indicates the number of infants for which data were available.

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

with application of the oxygen reduction test as previously described (19, 35, 52). Additional details on the method for assigning BPD status are provided in the Supplemental Material (see https://doi.org/10.5061/dryad.ns1rn8pnq). PH was defined as the presence of one of the following echocardiographic parameters: an estimated right ventricular systolic pressure (RVSP) greater than 40 mmHg, RVSP/systemic systolic blood pressure greater than 0.5, any cardiac shunt with bidirectional or right-to-left flow, or any degree of ventricular septal wall flattening. Serial echocardiograms were obtained to assess for PH at 7 days of age and at 36 wk PMA.

Data collection. In addition to clinical information, blood samples were collected at 7 days (± 48 h) after birth. Blood was placed into an EDTA-plasma tube, which was centrifuged after phlebotomy. The supernatant was removed, aliquoted, and stored at −80°C until assay. The blood sample collected closest to day seven was utilized for the primary analysis. More detail on the collected data is provided in the Supplemental Material.

Proteomic analyses. Proteomic analysis was conducted using the Slow Off-rate Modified Aptamer (SOMA) scan assay at the laboratories of SomaLogic (Boulder, CO) (40). This is a highly sensitive, quantitative, and reproducible proteomic tool that has been previously described in detail (14, 37, 41). At the time of assay, samples in each well of a 96-well plate were incubated with a mixture of the 1,121 SOMAmer reagents. Two sequential bead-based immobilization and washing steps eliminated unbound or nonspecifically bound proteins and the unbound SOMAmer reagents, leaving only protein target-bound SOMAmer reagents. These remaining SOMAmer reagents were isolated, and each reagent was quantified simultaneously on a custom Agilent hybridization array. The amount of each SOMAmer measured was quantitatively proportional to the protein concentration in the original sample. Additional information about the SOMAscan is provided in the Supplemental Material. The proteomic data will be made available to other researchers and will be accessible via the Gene Expression Omnibus (GEO) database (record no. GSE121097).

Statistical analysis. Descriptive statistics were calculated using means and standard deviations for continuous variables and frequencies and percentages for categorical variables. Demographic variables were compared across groups using ANOVA and chi-square or a Fisher's exact test, depending on the distribution of the variables.

All protein concentrations were log (base 2) transformed and compared according to BPD severity (none, mild, moderate, and severe) and by the presence of PH at 36 wk PMA. These comparisons were performed using linear contrasts of means in a two-way ANOVA with an interaction term for BPD severity and PH. *P* values were adjusted to control the false discovery rate (FDR) to account for the multiple tests performed on each protein; these adjustments were made within each a priori-specified comparison (i.e., BPD severe versus no BPD) (5). Both unadjusted and adjusted *P* values are presented. Random forests consisting of 5,000 classification trees were used to identify proteins that best discriminated across groups of interest (50). The random forest was performed in R using the RandomForest package (42).

In the final stage of the analysis, pathway analysis was conducted using pathways downloaded from Reactome (11, 31) and a functional

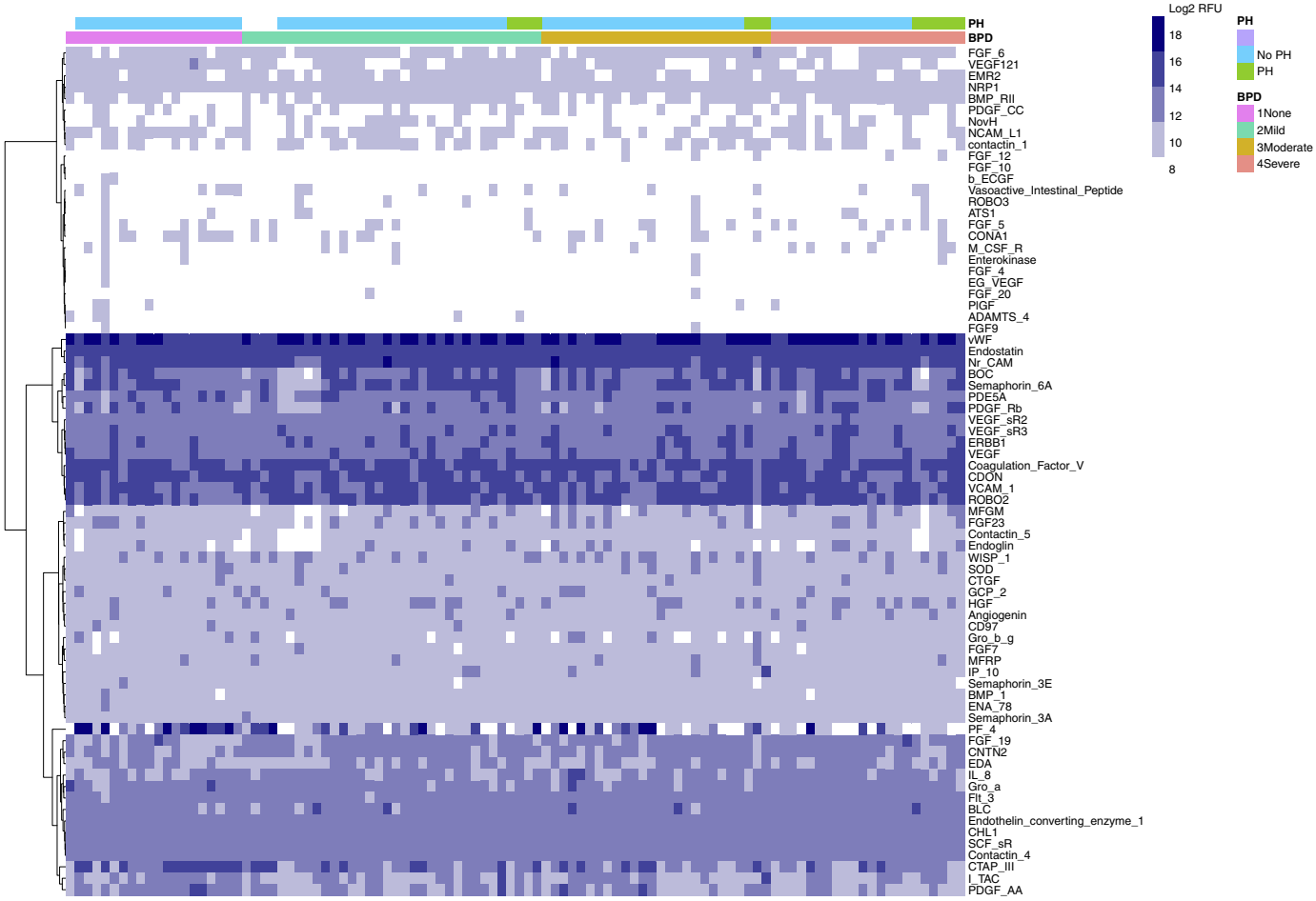

Fig. 1. Heatmap angiogenic proteins. Heatmap displaying the protein levels [log2 relative fluorescent units (RFU)] for each of the angiogenic factors (rows) by subject (columns). A darker color corresponds with a higher protein level. Columns are color coded by bronchopulmonary dysplasia (BPD) and pulmonary hypertension (PH) status at 36 wk postmenstrual age.

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

class scoring approach appropriate for platforms where proteins are selected a priori (21), using the *P* values as the protein-level statistics for the 4,001 proteins that were measured (39). This functional class scoring approach differs from an enrichment analysis in that it does not specifically test whether the pathways are enriched with a larger than expected number of significant proteins, and therefore also does not require a cutoff to be applied to each protein. The underlying inference from the functional class scoring approach is testing whether the pathway contains at least one measured protein that significantly differed between groups or whether a subset of proteins in the pathway have coordinated differences. Pathways were ranked based on the unadjusted *P* values, calculated using a permutation approach that appropriately accounts for the correlation between proteins, permuting group labels using 1,000 permutations (13). Spearman's rank-based correlations were calculated to describe the association between each pair of proteins across all samples. The matrix of correlations was then used in a network analysis using the igraph package in R.

In addition to the primary analysis, 76 blood samples were collected on days either before (days 3–5) or after (days 10–12) postnatal day 7 and, as a result, were excluded from the original analyses. The early plasma samples were from 64 individual infants; 26 infants were not included in the analyses of day 7. The late plasma samples were from 12 individual infants, of whom 4 infants were not included in the analyses of day 7. Therefore, we performed an analysis with these excluded blood samples for the identified top 10 proteins that were associated with development of BPD in the first week of life using ANOVA. The analysis of these contemporary close set of samples provides some assurance to substantiate the plausibility of the findings in the full set and to determine the consistency of results outside of an independent validation. All statistics were performed on data from infants with complete data and were computed using SAS version 9.4 and R (42, 43).

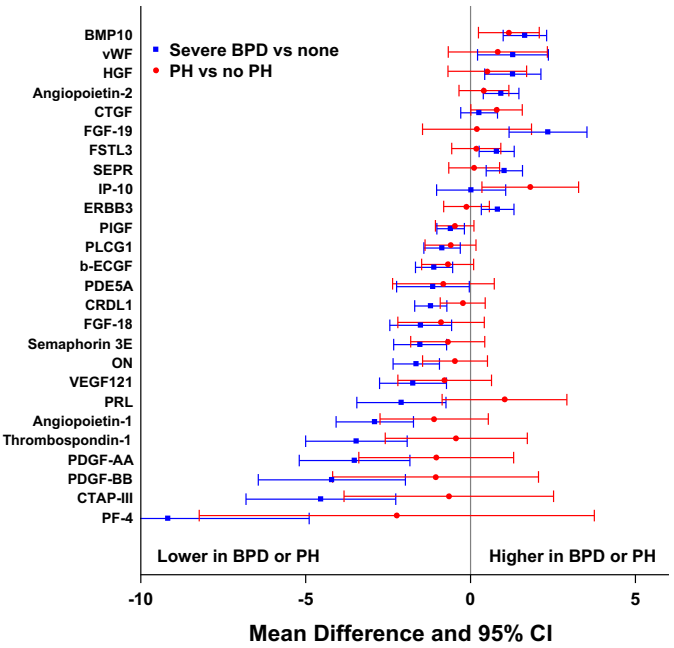

Fig. 2. Angiogenic peptides in bronchopulmonary dysplasia (BPD) and pulmonary hypertension (PH). Comparison of circulating levels of angiogenic peptides in preterm infants who develop severe BPD versus infants without BPD (blue) and infants with PH versus those without PH (red) at 36 wk postmenstrual age. The points and whiskers correspond to mean difference and 95% confidence interval (CI), respectively. The subset of the 94 angiogenic proteins with a CI that excludes 0 are plotted. BMP10, bone morphogenetic protein 10.

RESULTS

Eighty-two subjects with BPD and 20 infants who did not develop BPD were included in the study. Lower birth weight and gestational age were associated with the severity of BPD, and infants without BPD had lower rates of intubation and surfactant administration (Table 1). Thirteen subjects had PH at 36 wk PMA, 9 of whom also had moderate/severe BPD (Supplemental Fig. S1*A*). Early PH at day 7 was identified in 44 infants and was marginally associated with PH at 36 wk PMA (Supplemental Table S1).

Angiogenic factor proteins. Ninety-four of the proteins included in the SOMAscan were classified as angiogenic factors in the biological process gene ontology (Fig. 1 and Supplemental Table S2). As shown, several angiogenic proteins, including bone morphogenetic protein 10 (BMP10), von Willebrand factor (vWF), HGF, and angiopoietin 2 were most strongly increased, whereas PF-4, CTAP-III, PDGF-AA and PDGF-BB, prolactin (PRL), VEGF121, and others were significantly decreased in preterm infants who subsequently developed BPD (Fig. 2).

Although only 13 infants from this cohort were diagnosed with BPD and PH at 36 wk, we found that BMP10 was most strongly increased in infants with PH (Fig. 2). Overall, 20 protein levels differed when compared between severe BPD and no BPD, whereas only two differed between PH and no PH (Fig. 2). In general, the comparisons for BPD and PH were in the same direction, with the exception of PRL, which was increased in PH but decreased in severe BPD. The levels of CTAP-III, FGF-19, PDGF-AA, and PF-4 had a linear relationship between levels and severity of BPD (Fig. 3*A*, all linear trend *P* values <0.01; GEO Database record no. GSE121097). There was no difference in these proteins with PH, and there were marginal differences in BMP10, CTGF, IP-10, and PRL between infants with PH and those without PH (Fig. 3*B*). The top two ranked proteins for distinguishing across the 4 BPD categories using a random forest were FGF-19 and b-ECGF (Supplemental Fig. S2), and the classification error was 65.7%.

All SOMAmer protein targets. Comparisons of the 1,121 peptides between infants who developed severe BPD versus infants who did not develop BPD showed that 97 proteins differed between the 2 groups after FDR correction (Fig. 4). Top-ranked proteins included cystatin-M, elafin, and TARC. Results for all of the comparisons are included in the GEO Database (record no. GSE121097).

The top discriminating proteins from the random forest for infants across the 4 BPD severity groups were similar to those identified using the univariate analysis and included elafin and cystatin-M (Supplemental Fig. S3). The classification error from using all proteins was only slightly better than using the angiogenic factors alone (67.7%). There was a very low discriminative ability for PH as determined by the random forest approach using all proteins, and there was less overlap in the top-ranked proteins compared with the univariate approach than for the BPD severity comparisons (Supplemental Fig. S4). In addition, a four-group outcome using both BPD (none/mild versus moderate/severe) and PH was used. Not surprisingly, several of the top-ranked proteins for this comparison overlapped with the BPD-only outcome (Supplemental Fig. S5).

Pathway analyses. In addition to the proteins classified into the angiogenesis biologic process gene ontology, pathways

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

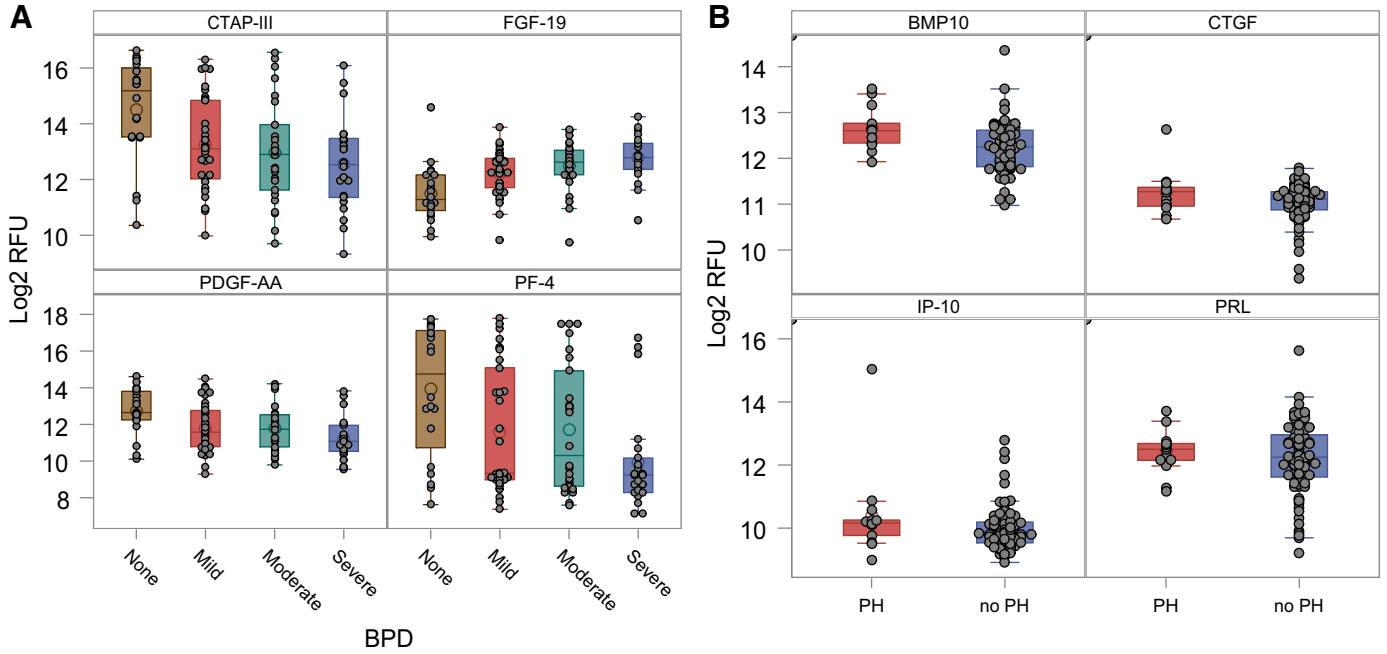

Fig. 3. Top 4 discriminating angiogenic proteins in bronchopulmonary dysplasia (BPD) severity and pulmonary hypertension (PH). Distribution of 4 angiogenic proteins that differ between preterm infants who subsequently developed severe BPD versus infants without BPD. Distribution within the other groups with BPD (*A*) and distribution with groups with BPD and PH (*B*) are included for comparison. The box indicates interquartile range (IQR) (25th–75th percentile) and the median and mean are indicated by the lines and large circles, respectively. Whiskers indicate data within 1.5 times the IQR, and points indicate individual data values. *P* values in BPD severity: CTAP-III, false discovery rate (FDR) $P = 0.02$; FGF-19, FDR $P = 0.02$; PDGF-AA, FDR $P = 0.01$; PF-4, FDR $P = 0.02$. *P* values PH versus no PH: bone morphogenetic protein 10 (BMP10), raw *P* value = 0.01 (FDR $P = 0.78$); CTGF, raw *P* value = 0.05 (FDR $P = 0.93$); IP-10 raw *P* value = 0.02 (FDR $P = 0.78$); PFRL raw *P* value = 0.28 (FDR $P = 0.93$). RFU, relative fluorescent units.

were evaluated for their association with increasing BPD severity and included cell surface interaction at the vascular wall, G protein-coupled receptor (GPCR) ligand binding, matrix remodeling, growth factor signaling and complement pathways (Table 2).

Network analysis. The network analysis indicated that there is a strong association between BMP10 and nonangiogenic peptides that were identified as being associated with severe BPD (Fig. 5*A*). Similarly, BMP10 was also highly correlated with other top-ranked proteins associated with PH. (Fig. 5*B*) In Fig. 5C, there is minimal agreement between the networks associated with BPD and PH, with the exception of a few proteins, including BMP10.

Adjusted for clinical factors. The main analysis focused on the discrimination of circulating protein levels to better understand the pathogenesis of BPD and PH. As noted in Table 1, some clinical factors are associated with the development of BPD and PH; however, these tend to not be modifiable risk factors. To better understand the contribution of the protein levels after including clinical factors, a random forest was fit with all proteins, gestational age, birth weight *z*-score, sex, and early PH (PH at 7 days PMA). All top-ranked factors were circulating protein levels, with the exception of gestational age, and were consistent with the random forest excluding clinical factors (Supplemental Fig. S6). The classification error rate for 4-level BPD severity increased to 69.0%. Higher cystatin-M, elafin, and macrophage mannose receptor and lower cathepsin A were associated with increasing BPD severity after adjusting for clinical risk factors (Supplemental Fig. S7).

Verification of results from analyses of excluded plasma samples. In total, 64 and 12 plasma samples before and after the day 7 collection window, respectively, were available to determine whether the findings in the main analysis were consistent with a contemporaneous set of samples (Supplemental Fig. S8). Analyses from the top 10 associated proteins verified that cystatin-M, chordin-like protein 1 (CRDL1), RET, and BMP10 in the plasma samples before day 7 and that cystatin-M, elafin, CRDL1, and BMP10 in the plasma samples after day 7 remained associated with BPD (Supplemental Table S3).

DISCUSSION

BPD persists as a major sequel of premature birth, causing early and late respiratory and cardiovascular morbidities. Despite major advances in care, the incidence of BPD has not changed over the past few decades, and preventive care strategies are limited, reflecting the importance of developing novel strategies for early identification of at-risk subjects. Based on strong preclinical and clinical studies that demonstrate an important role of disrupted angiogenesis in the pathogenesis of BPD and related PH, this study sought to determine if early changes in low-abundance circulating proteins related to angiogenesis could help identify preterm infants who are at high risk for developing BPD or BPD with PH. We used an extensive and unbiased large-scale, aptamer-based proteomic platform from prospectively collected plasma samples in preterm infants during the first week of life.

We found several peptides associated with angiogenic signaling that differed significantly between neonates who subse-

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

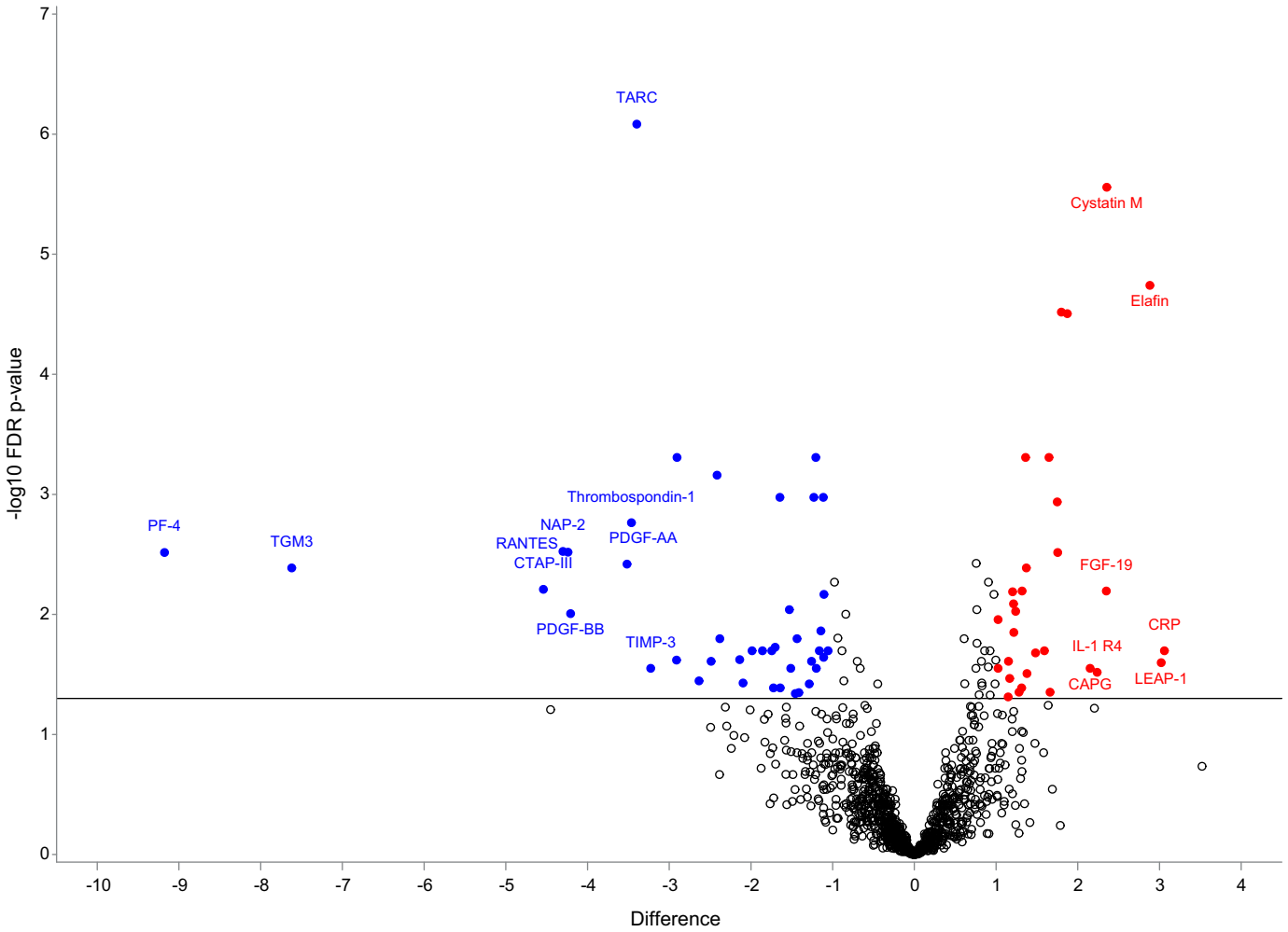

Fig. 4. Volcano plot of all proteins. Volcano plot displays the −log false discovery rate (FDR)-adjusted P values versus difference for the severe bronchopulmonary dysplasia (BPD) versus no BPD comparison. Proteins with an FDR value of $P < 0.05$ and an absolute difference of 3 or greater, where a difference of 1 on $\log_2$ scale corresponds to a fold change of 2, are labeled in the figure.

quently developed severe BPD in comparison with those without BPD; the peptides included increased levels of BMP10, vWF, hepatocyte growth factor (HGF), angiopoietin-2, FGF-19, and HGF and decreased levels of CRDL1, angiopoietin-1, ON, thrombospondin-1, b-ECGF, PF-4, PDGF-AA, PRL, PDGF-BB, VEGF121, FGF-18, and phospholipase C-γ-1 (PLCG1). In addition, several peptides not included in the list of angiogenic peptides were identified that best distinguished risk for BPD, including cystatin-M and elafin. Finally, we further found that increasing FGF-19 and decreasing PF-4, CTAP-III, and PDGF-AA levels were specifically associated with increasing grades of BPD severity.

We further analyzed the association of proteomic findings with PH, which was partially limited by the relatively small number of infants in the cohort who had PH at 36 wk. However, as a preliminary study, proteins related to angiogenic signaling differed significantly between the infants who developed PH at 36 wk PMA and the infants who did not. These proteins included increased levels of BMP10, which is involved in vascular development through the BMP pathway, and IP-10, which plays a role in the negative regulation of angiogenesis. Top-ranked nonangiogenic proteins that were involved in other processes (e.g., inflammatory), included increased levels of IL-2 receptor subunit-α and RANK, and decreased levels of CK-BB. We further found 5 peptides that differed in infants who developed PH and in infants who developed severe BPD (increased levels of BMP10, FCN1, TFF3, Marapsin, and DYRK3).

These findings are interesting because measurements of proteins that are associated with BPD may enable the earlier identification of patients at risk for BPD and development of novel preventive strategies. These data more specifically support the concept that early pulmonary vascular disease, as reflected by changes in angiogenic signals, is associated with high risk for the subsequent development of BPD. As pulmonary vessels enhance normal alveolar development, disruption of angiogenic signaling may alter pulmonary vessel development, which can impair alveolar growth and reduce lung surface area (1, 18, 47). Past clinical studies have shown that antenatal factors, such as maternal smoking, hypertension, gestational diabetes, and other causes of placental dysfunction, that often lead to fetal intrauterine growth restriction are strong determinants of poor respiratory outcomes in preterm infants (20, 30, 34, 48). Interestingly, Mestan et al. (30) reported that

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

Table 2. *Pathways*

Rank	Pathway Name	Number of SOMAmers in Pathway	Number of "Angiogenic" SOMAmers	Pathway *P* Value Unadjusted	Pathway *P* Value With No False Positive
1	Cell surface interactions at the vascular wall	45	2 (4.4%)	0.001	0.207
2	Chemokine receptors bind chemokines	24	9 (37.5%)	0.001	0.207
3	Class A/1 (rhodopsin-like receptors)	48	11 (22.9%)	0.001	0.207
4	$G\alpha_i$ signaling events	40	9 (22.5%)	0.001	0.207
5	GPCR ligand binding	61	14 (23.0%)	0.001	0.207
6	Peptide ligand-binding receptors	48	11 (22.9%)	0.001	0.207
7	Platelet degranulation	54	9 (16.7%)	0.001	0.207
8	Regulation of IGF transport and uptake by IGFBPs	64	3 (4.7%)	0.001	0.207
9	Response to elevated platelet cytosolic Ca^{2+}	57	9 (16.8%)	0.001	0.207
10	Post-translational protein phosphorylation	49	3 (6.1%)	0.001	0.245
11	Interleukin-4 and 13 signaling	55	5 (9.1%)	0.001	0.249
12	Interleukin-10 signaling	31	3 (9.7%)	0.001	0.294
13	Metabolism of lipids	41	1 (2.4%)	0.001	0.299
14	RET signaling	12	0	0.001	0.332
15	Signaling by PDGF	18	3 (16.7%)	0.001	0.332
16	Nonintegrin membrane-ECM interactions	14	1 (7.1%)	0.001	0.351
17	Signaling by TGF-β family members	18	1(5.6%)	0.001	0.353
18	DAP12 interactions	13	0	0.001	0.357
19	Complement cascade	36	0	0.001	0.357
20	Initial triggering of complement	20	0	0.001	0.362
21	Amyloid fiber formation	16	1 (6.3%)	0.001	0.370
22	VEGFR2-mediated cell proliferation	13	3 (23.1%)	0.001	0.402
23	Downstream signaling of activated FGFR4	18	7 (38.9%)	0.001	0.402
24	Phospholipase C-mediated cascade; FGFR4	15	7 (46.7%)	0.001	0.406
25	Signaling by ERBB2	19	1 (5.3%)	0.001	0.427
26	MHC class II antigen presentation	13	0	0.001	0.429
27	SHC-mediated cascade; FGFR4	16	7 (43.8%)	0.001	0.451
28	Gastrin-CREB signaling pathway via PKC and MAPK	23	2 (8.7%)	0.001	0.452
29	FRS-mediated FGFR4 signaling	16	7 (43.8%)	0.001	0.467
30	Dissolution of fibrin clot	11	0	0.001	0.473

ECM, extracellular matrix; FGFR4, fibroblast growth factor receptor 4; IGFBP, insulin-like growth factor-binding protein; MHC, major histocompatibility complex; SOMAmer, Slow Off-rate Modified Aptamer; TGF, transforming growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

decreases in key angiogenic proteins in cord blood of preterm infants, including VEGF-A and PLGF, strongly reflected abnormal placental vascular changes and were further associated with BPD and PH risk, further supporting the hypothesis that early impairment of angiogenesis is associated with, and likely contributes to, respiratory outcomes in preterm infants.

The top associated peptides are proteins involved in the inflammatory, cell development, and angiogenesis processes. Their biological functions and pathways are shown in Supplemental Table S4, *A* and *B*. In addition to the angiogenic peptides, proteins from other signaling pathways were identified with high risk for BPD, including GPCR signaling, matrix remodeling, growth factor signaling, and developmental pathways. We also found that peptides linked with angiogenesis are also linked with proteins included in other pathways, indicating that multiple mechanisms are likely involved in the pathophysiology of both BPD and PH, as reflected in Fig. 5. Additionally, we identified several proteins, including cystatin-M, CRDL1, RET, elafin, and BMP10, that were consistently altered during the first days after birth in samples excluded from the primary analysis, providing some degree of corroboration of our findings. However, only 2 proteins were consistently different in the plasma samples after day 7, but this analysis is limited by the small number of blood samples ($n = 12$) for comparisons. Despite these limitations, these pilot observations are interesting and may suggest that mechanisms impacting the development of BPD are most critical during the first week after birth.

Previous studies have shown that early disruption of angiogenesis in the developing lung impairs alveolarization and causes sustained abnormalities of lung structure that mimic clinical BPD (1, 8, 10, 18, 46). It has been shown that decreased proangiogenic and increased antiangiogenic factors are associated with high risk for the development of BPD (28). Factors identified included VEGF (increased in tracheal fluid during first postnatal days), circulating sFlt-1 and endostatin (increased in blood during first days of life), but also decreased cord blood markers such as endothelial progenitor cells, PLGF, granulocyte-colony stimulating factor (G-CSF), and VEGF-A. Each of these markers are involved in angiogenesis, and therefore, if disturbed, may cause abnormal development of the vasculature and reduce alveolarization in the preterm lung. In addition, each of these biomarkers has been associated with the subsequent development of BPD (9, 26, 30). Autopsy studies confirmed decreased levels of VEGF and increased levels of sFlt-1 by immunohistochemistry of the lungs of deceased infants with BPD (8, 10). As previously observed in a past study of cord blood samples (30), we found that circulating VEGF-121 and PLGF levels were decreased in the first week of life in preterm infants who subsequently developed severe BPD. We also found that G-CSF was significantly increased in infants with moderate/severe BPD versus infants with mild BPD or no BPD. We did not find significant differences between the protein levels of sFlt-1 and endostatin in infants with BPD. In addition, we found several other angiogenic peptides that were strongly associated with the development of PH and BPD and its severity.

Interestingly, among the nearly 1,200 peptides studied, early changes in circulating BMP10 levels were most strikingly

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

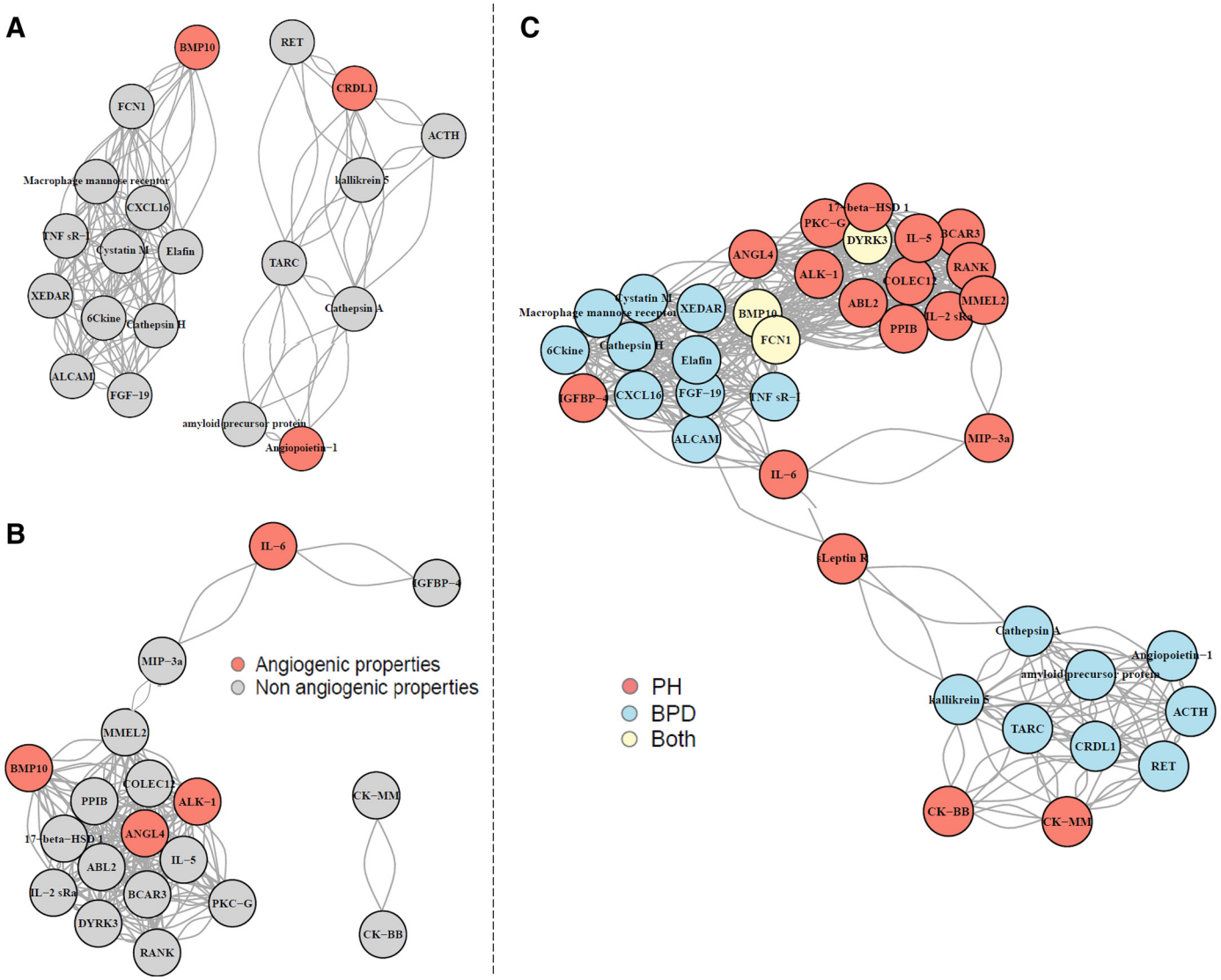

Fig. 5. Network figure of top identified proteins. Shown are the top angiogenic and nonangiogenic identified proteins. *A*: comparison between infants with severe bronchopulmonary dysplasia (BPD) and infants without BPD. *B*: comparison between infants with pulmonary hypertension (PH) at 36 wk postmenstrual age and infants without PH. *C*: proteins of comparisons *A* and *B* together. As shown, bone morphogenetic protein 10 (BMP10), a known angiogenic factor, is strongly correlated with several of the top 10 proteins from the discovery analysis. The results from this figure indicate that the top nonangiogenic proteins identified are highly correlated with at least one angiogenic protein, providing further evidence for the a priori hypothesis.

linked with the development of both BPD and BPD with PH. BMP10 is a high-affinity ligand for activin receptor-like kinase 1 (ALK1), a type I BMP receptor strongly expressed on endothelial cells, which has been implicated in diverse vascular diseases, including idiopathic and heritable forms of pulmonary arterial hypertension, hereditary hemorrhagic telangiectasia, and tumor angiogenesis (26a). Our findings further suggest that future studies of BMP10 as a biomarker and as a potential modulator of the pathobiology of BPD and BPD-associated PH are warranted.

We also identified other peptides within the angiogenic process to be associated with the development of BPD and its severity. This association may indicate that the early presence of pulmonary vascular disease could be associated with the development of BPD (32, 35). However, while some peptides were statistically associated with BPD severity (as shown in Fig. 3), the distributions overlap other severity groups, suggesting that protein levels may not necessarily discriminate between severity groups. Additionally, some of the identified angiogenic peptides (such as PDGF-AA and PDGF-BB, which play important roles during normal lung vascular development, and PF-4, which is a negative regulator of angiogenesis) were also found to differ in infants with pulmonary vascular disease at day 7 in comparison to infants without pulmonary vascular disease at day 7 from plasma samples of infants in the same cohort (51). Four of the top 20 peptides defined by the random forest analysis are associated with the angiogenesis, indicating that this process plays a significant role in the development of BPD, in addition to inflammatory pathways.

Recently, a small cohort study of preterm infants identified 12 peptides that were associated with the development of BPD (12). Three of the identified peptides (ANGPTL3, FGF-19, and EPHA2) are related to angiogenesis, further supporting the theory that early disruption of angiogenic factors may be

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

associated with the subsequent development of BPD. Additionally, peptides that are related to inflammation, cell growth, and the development of the nervous system were identified. Two of these 12 identified peptides, FGF-19 and TAJ, we also identified to be significantly different between infants with BPD and no BPD. FGF-19 is involved in embryonic development, cell growth, differentiation, and angiogenesis and is required for proper morphogenesis of the cardiac outflow tract. We speculate that FGF-19 may play a previously unrecognized role in the development of BPD (17).

A strength of our study is the use of the SOMAmer platform, which allows for the extensive and unbiased study of a large number of peptides. However, it also limited our ability to choose a more complete set of peptides for interrogation a priori. Therefore, other peptides that play a role in the pathogenesis of BPD and PH could be missed. Despite this, the angiogenic proteins that we investigated did reveal strong associations with the development of BPD and PH and supports the overall concept that altered angiogenic signaling is associated with high risk for BPD. In addition, developing specific signature panels utilizing the SOMAmer method may provide an exciting new technology for screening preterm infants for BPD and/or PH risk in the future.

As with all proteomic studies, a potential limitation of this study is that the levels of circulating proteins may not represent expression in lung tissue. Identified peptides are often multifunctional and belong to several different pathways; however, these peptides may stimulate novel preclinical research to develop greater insights into the pathobiology of BPD and PH. Additional limitation is the relatively small sample size of BPD infants with PH and the lack of a large independent validation cohort. However, despite the study having a small sample size, it is one of the largest prospective cohorts with extremely premature infants, and large amount of sample would be needed for validation studies, which are not available in this cohort. Analysis of plasma samples collected outside the day 7 window of our study was performed to verify the results from the main analysis. Finally, since the diagnosis of BPD is made at the age of 36 wk PMA, we could not assign a formal BPD diagnosis in infants who died early in the study. It is possible that we may have missed inclusion of infants with the most severe injury and arrest of lung development.

In summary, we found evidence that early changes in circulating proteins after preterm birth that reflect altered angiogenic signaling are associated with the subsequent development of BPD and PH at 36 wk PMA. These findings support the "vascular hypothesis" of BPD, in which early disruption of vascular growth and signaling contributes to the pathogenesis of BPD and that early events are critical determinants of disease inception. These data will enable further research in understanding the pathophysiology of BPD and PH in an early stage. A better understanding of the pathophysiology will eventually lead to an earlier identification of at-risk patients, better treatment strategies and, therefore may improve survival. We further found novel circulating biomarkers in preterm infants that are strongly associated with developing BPD and BPD with PH, such as BMP10, which may enhance early identification of at-risk preterm newborns and provide novel targets for future preclinical and clinical studies for disease prevention.

ACKNOWLEDGMENTS

The authors are grateful for the help of Gregory Seedorf, BS, for laboratory support and for specimen handling and preparation throughout the study.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-085703, "Genetic Basis for Impaired Angiogenesis in BPD" (S. H. Abman, PI), and HL-145679, "Physiologic Phenotyping of Respiratory Outcomes in Infants Born Premature" (R. Tepper and S. H. Abman, Mult-PIs) and by the NIH National Center for Research Resources Grant NCRR 1 K23 RR021921 "Pathogenetic Mechanisms of Bronchopulmonary Dysplasia" (P. M. Mourani, PI).

DISCLOSURES

S. H. Abman reports the following relationships with industry that are not related to this current work: laboratory research grants from United Therapeutics, Shire Pharmaceuticals, and Actelion. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

S.A., B.D.W., P.M.M., E.W.M., B.B.P., R.M.F.B., and S.H.A. conceived and designed research; S.A., B.D.W., and P.M.M. analyzed data; S.A., B.D.W., P.M.M., E.W.M., B.B.P., R.M.F.B., and S.H.A. interpreted results of experiments; S.A., B.D.W., and P.M.M. prepared figures; S.A., B.D.W., P.M.M., E.W.M., B.B.P., R.M.F.B., and S.H.A. drafted manuscript; S.A., B.D.W., P.M.M., E.W.M., B.B.P., R.M.F.B., and S.H.A. edited and revised manuscript; S.A., B.D.W., P.M.M., E.W.M., B.B.P., R.M.F.B., and S.H.A. approved final version of manuscript.

REFERENCES

1. **Abman SH.** Bronchopulmonary dysplasia: "a vascular hypothesis". *Am J Respir Crit Care Med* 164: 1755–1756, 2001. doi:10.1164/ajrccm.164.10.2109111c.
2. **Ambalavanan N, Morty RE.** Searching for better animal models of BPD: a perspective. *Am J Physiol Lung Cell Mol Physiol* 311: L924–L927, 2016. doi:10.1152/ajplung.00355.2016.
3. **Arjaans S, Zwart EAH, Ploegstra MJ, Bos AF, Kooi EMW, Hillege HL, Berger RMF.** Identification of gaps in the current knowledge on pulmonary hypertension in extremely preterm infants: A systematic review and meta-analysis. *Paediatr Perinat Epidemiol* 32: 258–267, 2018. doi:10.1111/ppe.12444.
4. **Baraldi E, Filippone M.** Chronic lung disease after premature birth. *N Engl J Med* 357: 1946–1955, 2007. doi:10.1056/NEJMra067279.
5. **Benjamini Y, Hochberg Y.** Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* 57: 289–300, 1995.
6. **Berenz A, Vergales JE, Swanson JR, Sinkin RA.** Evidence of early pulmonary hypertension is associated with increased mortality in very low birth weight infants. *Am J Perinatol* 34: 801–807, 2017. doi:10.1055/s-0037-1598246.
7. **Bhat R, Salas AA, Foster C, Carlo WA, Ambalavanan N.** Prospective analysis of pulmonary hypertension in extremely low birth weight infants. *Pediatrics* 129: e682–e689, 2012. doi:10.1542/peds.2011-1827.
8. **Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM.** Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in human infants dying with bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 164: 1971–1980, 2001. doi:10.1164/ajrccm.164.10.2101140.
9. **Borghesi A, Massa M, Campanelli R, Bollani L, Tzialla C, Figar TA, Ferrari G, Bonetti E, Chiesa G, de Silvestri A, Spinillo A, Rosti V, Stronati M.** Circulating endothelial progenitor cells in preterm infants with bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 180: 540–546, 2009. doi:10.1164/rccm.200812-1949OC.
10. **De Paepe ME, Greco D, Mao Q.** Angiogenesis-related gene expression profiling in ventilated preterm human lungs. *Exp Lung Res* 36: 399–410, 2010. doi:10.3109/01902141003714031.
11. **Fabregat A, Sidiropoulos K, Garapati P, Gillespie M, Hausmann K, Haw R, Jassal B, Jupe S, Korninger F, McKay S, Matthews L, May B, Milacic M, Rothfels K, Shamovsky V, Webber M, Weiser J, Williams M, Wu G, Stein L, Hermjakob H, D'Eustachio P.** The Reactome

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

pathway Knowledgebase. *Nucleic Acids Res* 44, D1: D481–D487, 2016. doi:10.1093/nar/gkv1351.

12. **Förster K, Sass S, Ehrhardt H, Mous DS, Rottier RJ, Oak P, Schulze A, Flemmer AW, Gronbach J, Hübener C, Desai T, Eickelberg O, Theis FJ, Hilgendorff A.** Early identification of bronchopulmonary dysplasia using novel biomarkers by proteomic screening. *Am J Respir Crit Care Med* 197: 1076–1080, 2018. doi:10.1164/rccm.201706-1218LE.
13. **Freedman D, Lane D.** A nonstochastic interpretation of reported significance levels. *J Bus Econ Stat* 1: 292–298, 1983. doi:10.2307/1391660.
14. **Ganz P, Heidecker B, Hveem K, Jonasson C, Kato S, Segal MR, Sterling DG, Williams SA.** Development and validation of a protein-based risk score for cardiovascular outcomes among patients with stable coronary heart disease. *JAMA* 315: 2532–2541, 2016. doi:10.1001/jama.2016.5951.
15. **Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG.** Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 42: 377–381, 2009. doi:10.1016/j.jbi.2008.08.010.
16. **Hilgendorff A, Reiss I, Ehrhardt H, Eickelberg O, Alvira CM.** Chronic lung disease in the preterm infant. Lessons learned from animal models. *Am J Respir Cell Mol Biol* 50: 233–245, 2014. doi:10.1165/rcmb.2013-0014TR.
17. **Itoh N, Ohta H, Nakayama Y, Konishi M.** Roles of FGF signals in heart development, health, and disease. *Front Cell Dev Biol* 4: 110, 2016. doi:10.3389/fcell.2016.00110.
18. **Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, Abman SH.** Inhibition of angiogenesis decreases alveolarization in the developing rat lung. *Am J Physiol Lung Cell Mol Physiol* 279: L600–L607, 2000. doi:10.1152/ajplung.2000.279.3.L600.
19. **Jobe AH, Bancalari E.** Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 163: 1723–1729, 2001. doi:10.1164/ajrccm.163.7.2011060.
20. **Keller RL, Feng R, DeMauro SB, Ferkol T, Hardie W, Rogers EE, Stevens TP, Voynow JA, Bellamy SL, Shaw PA, Moore PE, Alexander B, Chougnet C, Gratton T, Greenberg JM, Grisby C, Jobe AH, Koch B, McDowell K, Thornton K, Bates P, Cleveland C, Hamvas A, Hoffmann J, Holland MR, Kemp J, Levy PT, Linneman L, Sicard-Su J, Simpson G. Prematurity and Respiratory Outcomes Program.** Prematurity and respiratory outcomes program. Bronchopulmonary dysplasia and perinatal characteristics predict 1-Year respiratory outcomes in newborns born at extremely low gestational age: a prospective cohort study. *J Pediatr* 187: 89–97.e3, 2017. doi:10.1016/j.jpeds.2017.04.026.
21. **Khatri P, Sirota M, Butte AJ.** Ten years of pathway analysis: current approaches and outstanding challenges. *PLOS Comput Biol* 8: e1002375, 2012. doi:10.1371/journal.pcbi.1002375.
22. **Khemani E, McElhinney DB, Rhein L, Andrade O, Lacro RV, Thomas KC, Mullen MP.** Pulmonary artery hypertension in formerly premature infants with bronchopulmonary dysplasia: clinical features and outcomes in the surfactant era. *Pediatrics* 120: 1260–1269, 2007. doi:10.1542/peds.2007-0971.
23. **Kim GB.** Pulmonary hypertension in infants with bronchopulmonary dysplasia. *Korean J Pediatr* 53: 688–693, 2010. doi:10.3345/kjp.2010.53.6.688.
24. **König K, Guy KJ, Nold-Petry CA, Barfield CP, Walsh G, Drew SM, Veldman A, Nold MF, Casalaz DM.** BNP, troponin I, and YKL-40 as screening markers in extremely preterm infants at risk for pulmonary hypertension associated with bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* 311: L1076–L1081, 2016. doi:10.1152/ajplung.00344.2016.
25. **Lal CV, Ambalavanan N.** Biomarkers, early diagnosis, and clinical predictors of bronchopulmonary dysplasia. *Clin Perinatol* 42: 739–754, 2015. doi:10.1016/j.clp.2015.08.004.
26. **Lassus P, Turanlahti M, Heikkilä P, Andersson LC, Nupponen I, Sarnesto A, Andersson S.** Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. *Am J Respir Crit Care Med* 164: 1981–1987, 2001. doi:10.1164/ajrccm.164.10.2012036.

26a. **Li W, Salmon RM, Jiang H, Morrell NW.** Regulation of the ALK1 ligands, BMP9 and BMP10. *Biochem Soc Trans* 44: 1135–1141, 2016. doi:10.1042/BST20160083.

27. **Lynch AM, Wagner BD, Deterding RR, Giclas PC, Gibbs RS, Janoff EN, Holers VM, Santoro NF.** The relationship of circulating proteins in early pregnancy with preterm birth. *Am J Obstet Gynecol* 214: 517.e1–517.e8, 2016. doi:10.1016/j.ajog.2015.11.001.
28. **Mandell EW, Abman SH.** Fetal vascular origins of bronchopulmonary dysplasia. *J Pediatr* 185: 7–10.e1, 2017. doi:10.1016/j.jpeds.2017.03.024.
29. **McEvoy CT, Jain L, Schmidt B, Abman S, Bancalari E, Aschner JL.** Bronchopulmonary dysplasia: NHLBI workshop on the primary prevention of chronic lung diseases. *Ann Am Thorac Soc* 11, *Suppl* 3: S146–S153, 2014. doi:10.1513/AnnalsATS.201312-424LD.
30. **Mestan KK, Gotteiner N, Porta N, Grobman W, Su EJ, Ernst LM.** Cord blood biomarkers of placental maternal vascular underperfusion predict bronchopulmonary dysplasia-associated pulmonary hypertension. *J Pediatr* 185: 33–41, 2017. doi:10.1016/j.jpeds.2017.01.015.
31. **Milacic M, Haw R, Rothfels K, Wu G, Croft D, Hermjakob H, D'Eustachio P, Stein L.** Annotating cancer variants and anti-cancer therapeutics in reactome. *Cancers (Basel)* 4: 1180–1211, 2012. doi:10.3390/cancers4041180.
32. **Mirza H, Ziegler J, Ford S, Padbury J, Tucker R, Laptook A.** Pulmonary hypertension in preterm infants: prevalence and association with bronchopulmonary dysplasia. *J Pediatr* 165: 909–14.e1, 2014. [Erratum in *J Pediatr* 166: 782, 2015.] doi:10.1016/j.jpeds.2014.07.040.
33. **Montgomery AM, Bazzy-Asaad A, Asnes JD, Bizzarro MJ, Ehrenkranz RA, Weismann CG.** Biochemical screening for pulmonary hypertension in preterm infants with bronchopulmonary dysplasia. *Neonatology* 109: 190–194, 2016. doi:10.1159/000442043.
34. **Morrow LA, Wagner BD, Ingram DA, Poindexter BB, Schibler K, Cotten CM, Dagle J, Sontag MK, Mourani PM, Abman SH.** Antenatal determinants of increased risk for bronchopulmonary dysplasia and late respiratory disease in preterm infants. *Am J Respir Crit Care Med* 196: 364–374, 2017. doi:10.1164/rccm.201612-2414OC.
35. **Mourani PM, Sontag MK, Younoszai A, Miller JI, Kinsella JP, Baker CD, Poindexter BB, Ingram DA, Abman SH.** Early pulmonary vascular disease in preterm infants at risk for bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 191: 87–95, 2015. doi:10.1164/rccm.201409-1594OC.
36. **Mourani PM, Mandell EW, Meier M, et al.** Early PVD in preterm infants is associated with late respiratory outcomes in childhood. *Am J Respir Crit Care Med* 199: 1020–1027, 2018. doi:10.1164/rccm.201803-0428OC.
37. **Ngo D, Sinha S, Shen D, Kuhn EW, Keyes MJ, Shi X, Benson MD, O'Sullivan JF, Keshishian H, Farrell LA, Fifer MA, Vasan RS, Sabatine MS, Larson MG, Carr SA, Wang TJ, Gerszten RE.** Aptamer-based proteomic profiling reveals novel candidate biomarkers and pathways in cardiovascular disease. *Circulation* 134: 270–285, 2016. doi:10.1161/CIRCULATIONAHA.116.021803.
38. **Northway WH Jr, Rosan RC, Porter DY.** Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med* 276: 357–368, 1967. doi:10.1056/NEJM196702162760701.
39. **Poole W, Gibbs DL, Shmulevich I, Bernard B, Knijnenburg TA.** Combining dependent P-values with an empirical adaptation of Brown's method. *Bioinformatics* 32: i430–i436, 2016. doi:10.1093/bioinformatics/btw438.
40. **Reactome.** *Reactome Project 2012*. 10/17/2012. http://www.reactome.org.
41. **Rohloff JC, Gelinas AD, Jarvis TC, Ochsner UA, Schneider DJ, Gold L, Janjic N.** Nucleic acid ligands with protein-like side chains: modified aptamers and their use as diagnostic and therapeutic agents. *Mol Ther Nucleic Acids* 3: e201, 2014. doi:10.1038/mtna.2014.49.
42. **RStudio Team.** *RStudio: Integrated Development for R. RStudio, Inc*., Boston, MA. 2015. http://www.rstudio.com/.
43. **SAS Institute Inc.** *SAS version 9.4*. Cary, NC. 2014. https://www.sas.com.
44. **Seedorf G, Metoxen A, Rock R, Markham N, Ryan S, Vu T, Abman SH.** Hepatocyte growth factor as a downstream mediator of vascular endothelial growth factor-dependent preservation of growth in the developing lung. *Am J Physiol Lung Cell Mol Physiol* 310: L1098–L1110, 2016. doi:10.1152/ajplung.00423.2015.
45. **Smith VC, Zupancic JA, McCormick MC, Croen LA, Greene J, Escobar GJ, Richardson DK.** Rehospitalization in the first year of life among infants with bronchopulmonary dysplasia. *J Pediatr* 144: 799–803, 2004. doi:10.1016/j.jpeds.2004.03.026.
46. **Stenmark KR, Abman SH.** Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia. *Annu Rev Physiol* 67: 623–661, 2005. doi:10.1146/annurev.physiol.67.040403.102229.

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.

47. **Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, Hale EC, Newman NS, Schibler K, Carlo WA, Kennedy KA, Poindexter BB, Finer NN, Ehrenkranz RA, Duara S, Sánchez PJ, O'Shea TM, Goldberg RN, Van Meurs KP, Faix RG, Phelps DL, Frantz ID III, Watterberg KL, Saha S, Das A, Higgins RD; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network.** Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 126: 443–456, 2010. doi:10.1542/peds.2009-2959.
48. **Taglauer E, Abman SH, Keller R.** Recent advances in antenatal factors predisposing to bronchopulmonary dysplasia. *Semin Perinatol* 42: 413–424, 2018. doi:10.1053/j.semperi.2018.09.002.
49. **Thébaud B, Abman SH.** Bronchopulmonary dysplasia: where have all the vessels gone? Roles of angiogenic growth factors in chronic lung disease. *Am J Respir Crit Care Med* 175: 978–985, 2007. doi:10.1164/rccm.200611-1660PP.
50. **Touw WG, Bayjanov JR, Overmars L, Backus L, Boekhorst J, Wels M, van Hijum SA.** Data mining in the life sciences with random forest: a walk in the park or lost in the jungle? *Brief Bioinform* 14: 315–326, 2013. doi:10.1093/bib/bbs034.
51. **Wagner BD, Babinec AE, Carpenter C, Gonzalez S, O'Brien G, Rollock K, Williamson K, Mourani PM, Abman SH.** Proteomic profiles associated with early echocardiogram evidence of pulmonary vascular disease in preterm infants. *Am J Respir Crit Care Med* 197: 394–397, 2018. doi:10.1164/rccm.201703-0654LE.
52. **Walsh MC, Wilson-Costello D, Zadell A, Newman N, Fanaroff A.** Safety, reliability, and validity of a physiologic definition of bronchopulmonary dysplasia. *J Perinatol* 23: 451–456, 2003. doi:10.1038/sj.jp.7210963.
54. **Yun EJ, Lorizio W, Seedorf G, Abman SH, Vu TH.** VEGF and endothelium-derived retinoic acid regulate lung vascular and alveolar development. *Am J Physiol Lung Cell Mol Physiol* 310: L287–L298, 2016. doi:10.1152/ajplung.00229.2015.

Downloaded from journals.physiology.org/journal/ajplung at University of Groningen (129.125.058.118) on April 15, 2020.