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Early identification of Bronchopulmonary Dysplasia using novel biomarkers by proteomic screening

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Introduction

Bronchopulmonary Dysplasia (BPD) concerns up to 77% of all preterm infants and is notable for its significant long-term sequelae. Defined by the need for oxygen supplementation or ventilator support at term, early and quantifiable disease markers still remain elusive.

Our aim was therefore to identify and validate early plasma markers indicating BPD development with high sensitivity by the use of comprehensive protein screening.

Patients and Methods

Study population

35 preterm infants with informed parental consent and a GA ≤ 32 weeks were prospectively included in the study (table 1): Exploration cohort, Perinatal Center of the Ludwig-Maximilians-University, Campus Grosshadern (n=18, EC #195-07); independent confirmation cohort, Perinatal Center of the University Hospital Giessen (n=17, EC #135/12). Mild, moderate or severe BPD was diagnosed at 36 weeks (2001) together with the days of invasive and non-invasive mechanical ventilation and oxygen treatment. Sepsis was defined by presence of both clinical and laboratory findings (temperature instability, metabolic acidosis, cardiorespiratory instability, hyperglycemia, capillary refill time > 2s, CrP, interleukin 6, I/T ratio, WBC). Some cases were confirmed by pathogen detection from blood or cerebrospinal fluid. Chorioamnionitis was confirmed by placental histology (50%) or maternal/ fetal signs of infection at birth.

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Biomarker analysis

Serial plasma samples generated from whole blood EDTA specimen obtained in the first week of life (day 0-4 n=16, day 5-7 n=16) and at day 28 (n=14) were subjected to proteomic screening (SOMAscan™, SomaLogic, Boulder, USA). Protein binding to 1129 individual high affinity molecules (SOMAmer®) was quantified by custom Agilent hybridization array (1, 2) with high reproducibility even in low amount samples <100µl. Confirmation of protein expression in ELISA technique (SIGLEC-14 R&D systems; BCAM: Thermofischer Scientific; ANGPTL3 Raybiotech) used 1-2 samples from the first week of life.

Immunohistochemistry in preterm lung

Paraformaldehyde tissue sections from autopsy lungs of five BPD infants and an infant with a non-pulmonary cause of death (Sophia Children's Hospital, Erasmus Medical Center, Rotterdam; GA 26.6±2.0 weeks, MV 42±14 days, mean and SD each) demonstrated pulmonary protein expression: (SIGLEC-14, 1:50, #MAB10721, R&D systems; BCAM, 1:200, #sc-99188, Santa Cruz Biotech; ANGPTL3, 1:50,#600-401-Y15, Rockland; 2° antibody 1:300; Vector ABC reagent, DAB).

Statistical analysis

47 samples passed the SOMAscan™ quality control. Outliers were identified by principal component analysis (PCA, prcomp function, R framework, log-transformed data. First, to subtract confounding effects by considering low sample number we applied L1-regularized regression (Lasso) in each expression profile using the critical clinical variables presented in table 1 excluding disease outcome. Second, to identify disease-related biomarkers, we fitted a generalized additive model including disease information (BPD 0 vs. BPD 1, 2, 3) as binary variables, time points as spline effects

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and patients as random effects. Benjamini-Hochberg correction for multiple testing was applied. Gene Set Enrichment Analysis (GSEA, $p<0.1$) (3) was used with functional annotations from Gene Ontology (GO). Logistic regression model with forward model selection identified the minimal protein set best describing different BPD levels in the significantly associated proteins. Akaike information criterion (AIC) quantified the quality of each model thereby simultaneously accounting for predictive power and model complexity (4). Poisson regression was used to model the count variables days of oxygen supplementation and days of mechanical ventilation (MV) under the consideration of the clinical confounding variables (table 1).

Results

After correction for clinical confounding variables (table 1), twelve proteins were significantly regulated in preterm infants developing BPD **(Fig. 1A,** $p<0.1$, FDR 0.053 to 0.096), reflecting the GO categories identified by functional enrichment analysis, e.g. ‘immune function’, ‘defense response’, ‘extracellular matrix’ (ECM), ‘cellular proliferation’, ‘cellular migration’, and ‘organ development.

Increased detection levels for SIGLEC-14 and BCAM in combination with decreased levels for ANGPTL3 in the first week of life were found to describe the outcome variable ‘BPD’ with high specificity and sensitivity with model selection showing separation of disease groups **(Fig. 1B, C).** Analysis using the count variable ‘oxygen supplementation’ and ‘ventilator support’ confirmed the results. Protein levels remained stable at day 28 of life. Validation of the findings succeeded in a second, independent cohort using individual logistic regression **(Fig. 1D)**. Pulmonary expression of the proteins and their characteristic regulation was demonstrated in lung tissue sections from BPD patients **(Fig. 1E)**.

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Discussion

Our study establishes a validated set of proteins identifying BPD early after birth indicating key processes of disease development by the use of proteomic screening in preterm infants. Both, unbiased screening as well as validation experiments significantly add to previous studies using single markers lacking disease specificity and validation (5).

Out of the significantly regulated proteins closely reflecting BPD pathophysiology, early plasma levels of SIGLEC-14, BCAM and ANGPTL3 showed high sensitivity and specificity for BPD, exceeding the prognostic value of clinical variables currently used for risk stratification (6). Expressed on granulocytes and monocytes, SIGIEC-14 is attributed to invasive infections in newborns (8) and lung disease in adults (9) through pathogen-induced host immune suppression. BCAM, associated with impaired airway-branching and lung structure development, and ANGPTL3, playing a role in endothelial development and survival, likely indicate structural -term effects. The potential of the proteins to risk-stratify affected infants with BPD into respective disease-subtypes as well as their value as treatment targets need to be exploited further. The potential of the plasma proteins to serve as BPD biomarkers is supported by proving their pulmonary expression and demonstrating their stable expression levels in the later disease course.

Limitations of our study include the patient number that we however consider adequate for a pilot study and the fact that despite the prospective study design, patients cannot be considered randomly assigned to the disease groups. To overcome the limitations of disease grading, all critical findings from the study were confirmed by the use of the count variables ‘duration of mechanical ventilation’ and ‘duration of oxygen supplementation’ and the investigation of two independent

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cohorts. Despite significant differences between the two cohorts with respect to some clinical variables, the confirmation of the biomarkers emphasizes the robustness of the results.

In summary, our study is a first step towards an early diagnostic tool allowing for timely decision-making, disease monitoring and development of personalized treatment strategies in BPD as recommended (10).

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Table 1. Patient characteristics

	Exploration cohort	Confirmation cohort
n	**18**	**17**
Gestational age (weeks PMA)	26.2 (24.3-28.2)	26.2 (24.4-29.6)
Birth weight (g) *	755 (510-1040)	840 (340-1470)
IUGR *	4 (22.2%)	1 (5.9%)
Gender (female/male)	11/7	12/5
pH, umbilical artery	7.33 (6.95-7.47)	7.35 (7.01-7.48)
ANCS	17 (94.0%)	14 (82.4%)
Chorioamnionitis[1]	13 (72.0%)	12 (70.6%)
Clinical sepsis[2]	6 (33.3%)	4 (23.5%)
RDS ≥ grade 3[3]	9 (50.0%)	4 (23.5%)
Days of mechanical ventilation*	54 (33-78)	24 (2-74)
- Endotracheal mechanical ventilation (n/days)	6 (1-41)	2 (0-32)
- Pharyngeal ventilation/CPAP (n/days)	40.5 (30-55)	20 (2-52)
PDA	10 (55.6%)	12 (70.6%)
Postnatal corticosteroids *	11 (61.1%)	1 (5.9%)
ROP ≥ grade 3 *	0 (0.0%)	5 (29.4%)
IVH ≥ grade 3 *	0 (0.0%)	2 (11.8%)
ICU stay (days) *	78.5 (57-110)	38.0 (5-93)
BPD[4]		
- None	4 (22.2%)	3 (17.6%)
- Mild	8 (44.4%)	9 (52.9%)
- Moderate	2 (11.1%)	1 (5.9%)
- Severe	4 (22.2%)	4 (23.5%)

Data are given as median and range or number and percent of total in group. ANCS, Antenatal corticosteroids (two doses of betamethasone >24 hours before and no later than 7 days before birth). BPD, Bronchopulmonary Dysplasia; CPAP, continuous positive airway pressure; ICU, intensive care unit; IUGR, intrauterine growth retardation (birth weight <10th percentile); IVH, intraventricular haemorrhage; PDA, patent ductus arteriosus; PMA, post-menstrual age; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity. [1]Franz AR, et al. Acta Paediatr 2001; [2]Sherman MP et al. Pediatrics 1980 and http://www.nrz-hygiene.de/surveillance/kiss/neo-kiss/; [3]Couchard M et al. Ann Radiol 1974; [4]Jobe AH, Bancalari E. Am J Respir Crit Care Med 2001
* $p < 0.05$

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Fig. 1: (A) Identification of plasma biomarkers by proteomic screening. Results are displayed as scaled expression profiles after removal of confounder effects showing 12 proteins significantly regulated with BPD**. (B)** AIC analysis revealed a panel of three proteins with high sensitivity for BPD. For visualization the confounder effects (GA, gender, AIS, early onset infection, RDS, steroid treatment) were subtracted and protein expression was fitted into a linear model. BCAM, Siglec-14 and ANGPTL3 were found to separate the disease groups in the exploration cohort. **(C)** Sensitivity and specificity for the identification of BPD using three individual logistic regression models for BPD grades 1 (mild), 2 (moderate) and 3 (severe) compared to 0 (no) by BCAM, Siglec-14 and ANGPTL3 plasma concentration in the first week of life. **(D)** Confirmation of the biomarker expression by ELISA technique in a second, independent cohort (n=17 preterm infants). Individual logistic regression analysis (area under the ROC 0.93) confirmed the results for SIGLEC-14, BCAM, ANGPTL-3. **(E)** Immunostaining for the identified biomarker proteins in human preterm lungs indicating increased pulmonary expression of SILGEC-14 **(A)**, BCAM **(B)** and reduced ANGPTL3 **(C)** in preterm infants with mild, moderate or severe BPD as compared to an infant without BPD (control). Red arrows indicate positive stain. Magnification 400x.

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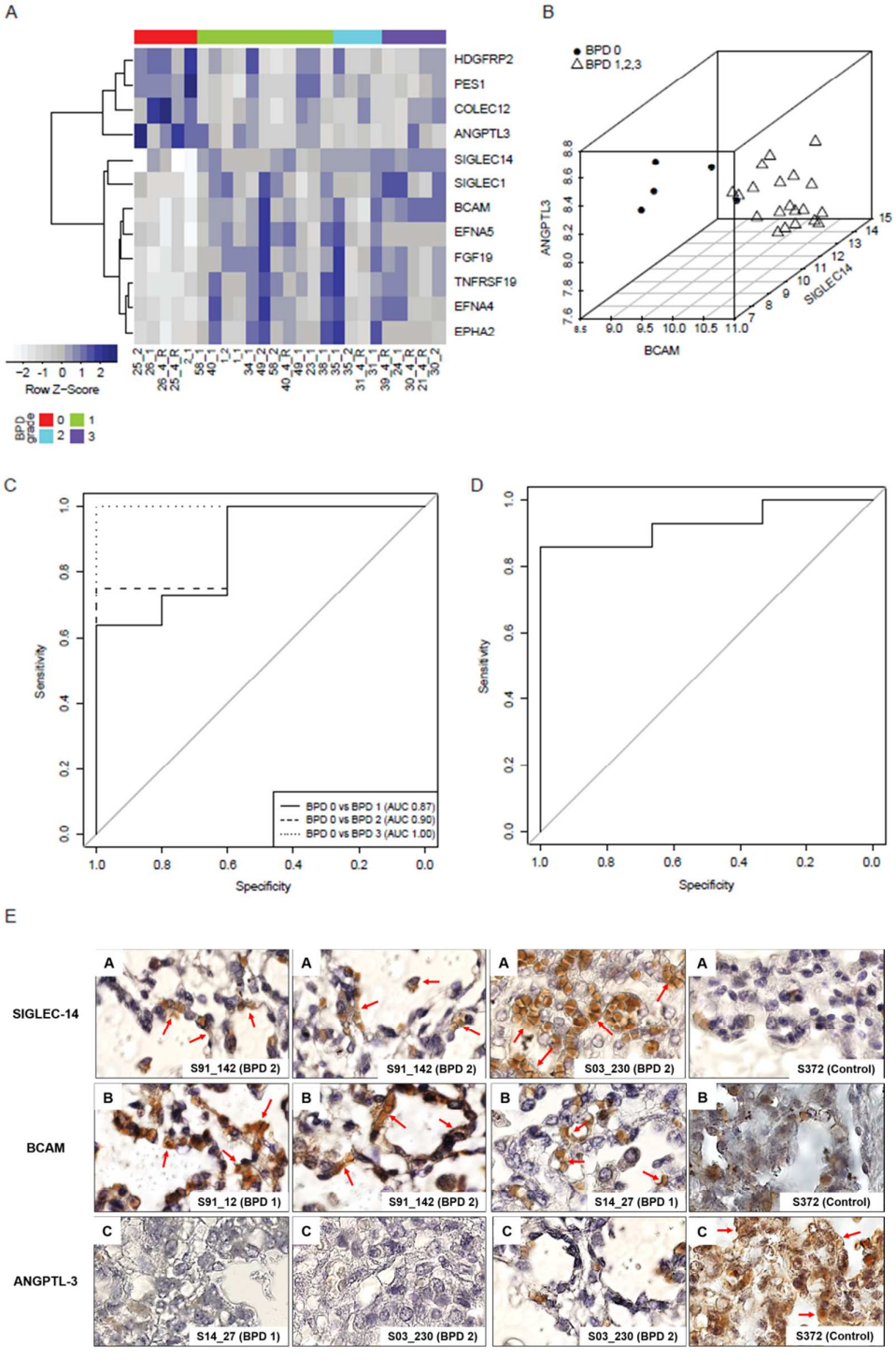

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