Clinical study protocol

Study title: Novel predictive indicators for bronchopulmonary dysplasia in extremely preterm infants

PIs/Protocol authors (alphabetic order): Tobias Werther[1], Lukas Wisgrill[1], Katharina Göral[1]

Co-authors (alphabetic order): Lukas Aichhorn[1], Francesco Cardona[1], Robyn Dvorsky[1], Katrin Klebermass-Schrehof[1], Michael Wagner[1], Anke Mertens[1]

[1] Department of Pediatrics and Adolescent Medicine, Division of Neonatology, Pediatric Intensive Care and Neuropediatrics, CCP, Medical University of Vienna, Vienna, Austria.

1 Study synopsis

Title: *Novel predictive indicators for bronchopulmonary dysplasia in extremely preterm infants*

Background: *Despite improvements in survival and morbidity rates among extremely preterm infants, the incidence of bronchopulmonary dysplasia (BPD), defined as the need for supplemental oxygen or respiratory support at 36 weeks' postmenstrual age, remains high.*

Aim of the study*: To assess the predictive value of emerging metrics from novel technologies in extremely preterm infants for BPD.*

Study design: *Prospective observational clinical trial.*

Participants: *Infants born below 26 weeks of gestation and admitted to the neonatal intensive care units of the Medical University of Vienna.*

Methods: *Prognostic indicators for BPD:*

1. *peak inspiratory pressure and expiratory tidal volume assessed with a Respiratory Function Monitor (RFM) during neonatal transition and resuscitation,*
2. *lung ultrasound score (LUS) and diaphragmatic excursion (DU) assessed by ultrasound in the first 28 days of life,*
3. *targeted neonatal echocardiography (TnEcho) assessed repetitively until* 36 weeks of postmenstrual age (PMA),
4. *respiratory system reactance assessed by the Forced Oscillation Technique (FOT) during the first 14 days of life and at 3 and 5 years of age,*
5. *peak and minimum electrical activity of the diaphragm assessed by Neurally-Adjusted Ventilatory Assist (NAVA) device in the first 14 days of life,*
6. *center of ventilation, overdistension and atelectasis/collapse, and global inhomogeneity index assessed by electrical impedance tomography (EIT) in the first 14 days of life,*
7. *proteomic analysis of plasma and tracheal aspirate samples collected at frequent intervals during the first 28 days of life.*

Statistical Analysis: *Novel predictive indicators will be correlated with the respiratory severity score (RSS) defined by the mean-airway pressure (MAP) times the fraction of inspired oxygen (FiO2). Uni- and multivariate logistic regressions will be performed to investigate the ability of the predictive indicators to prognosticate BPD. Receiver operating characteristic (ROC) analysis will*

be applied to identify the optimal cut-off value for each indicator in predicting BPD, and the C-statistics will be calculated to assess the predictive performance of our models.

Primary outcome: *1. Correlation between the novel predictive indicators and RSS.*

2. ROC analysis of each prognostic indicators to predict BPD.

Secondary outcome: *The ability to predict BPD using uni-variable logistic models for each predictive indicator. To clarify which parameter will be best to predict BPD using multivariable logistic models. Mutual correlations between the predictive indicators. Differences between LUS, DU, TnEcho, NAVA, EIT, and proteomic analysis (PA) performed in intubated and non-intubated patients; difference between longitudinal measurements (LUS, DU, TnEcho, FOT, NAVA, EIT, PA) performed in the first four weeks of life, difference of the indicators (LUS, DU, TnEcho, FOT, NAVA, EIT, PA) during routinely performed PEEP/CDP trials, differences between the indicators (LUS, DU, TnEcho, FOT, NAVA, EIT, PA) before, during and after treatments affecting lung function; survival rates and major morbidities of study participants.*

Number of subjects: *Based on available data (expected rate of BPD of 30%) and a dropout rate of 30%, the inclusion of 140 participants is planned.*

Risk/benefit*: All study participants will undergo intensive or intermediate medical care, with existing treatment regimens remaining unaltered. The data acquired will provide insights into the development of BPD and facilitate the optimization of lung-protective ventilation strategies.*

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3 Glossary

AUC	area under the curve	**RFM**	respiratory function monitoring
GA	gestational age	**ROC**	Receiver operating characteristic
PMA	postmenstrual age	**TnEcho**	targeted neonatal echocardiography
BPD	bronchopulmonary dysplasia	**RSS**	respiratory severity score
CDP	continuous distending pressure	**SD**	standard deviation
CPAP	continuous positive airway pressure	**SpO2**	peripheral oxygen saturation
CO2	carbon dioxide	**TET**	minimal diaphragmatic thickness
DO	first 30 min after birth	**TIT**	maximal diaphragmatic thickness
DN	period from the $(N-1)^{th}$ to $(N+1)^{th}$ the day of life	**TV**	tidal volume
DSF	diaphragm shortening fraction	**Xrs**	respiratory system reactance
DU	diaphragmatic ultrasound		
EIT	electrical impedance tomography		
FiO2	fraction of inspired oxygen		
FOT	forced oscillation technique		
HFOV	high frequency oscillatory ventilation		
LUS	lung ultrasound		
MAP	mean airway pressure		
NAVA	neurally adjusted ventilation assist		
NPLUS	neonatologist performed LUS		
PA	proteomic analysis		
pCO2	partial pressure of carbon dioxide		
PDA	patent ductus arteriosus		
PEEP	positive end-expiratory pressure		
PHT	pulmonary hypertension		
PIP	peak inspiratory pressure		
pO2	partial pressure of oxygen		

4 Scientific/Medical section

4.1 Background

Bronchopulmonary dysplasia (BPD) is one of the main complications in prematurely born infants and is associated with severe pulmonary and neurocognitive long-term sequelae.[1-3] Despite several advances in the peri- and postnatal care, the incidence of BPD remains high, reaching 20-40%.[4-6] Early identification of high-risk infants would allow for timely decision-making as well as individualized preventive and therapeutic interventions.[4]

Various studies proposed different mechanisms for the early “hits” leading to lung injury.[7] Excessive tidal volumes during newborn resuscitation, mechanical ventilation and high oxygen supplementation has been identified as major causes of lung damage and BPD development.[7-11] Early respiratory patterns of premature infants, defined by fraction of inspired oxygen (FiO2), are associated with an increased risk of developing BPD.[12] Oxygen requirement often ensues from low lung volume, which can be improved by applying positive end-expiratory pressure (PEEP). However, setting the optimal PEEP level is challenging (as lung volume surveillance at the bedside is still an unsolved problem). Elevated PEEP levels, although improving oxygenation, may be harmful by (partially) overinflating the lung and, in turn, provoking lung injury including air leaks. This problem persists for both non-invasive and invasive respiratory set-ups. Therefore, minimizing FiO2 by applying high PEEP may not be optimal to prevent BPD. Insofar, while FiO2 represents a risk factor, it may not serve as a predictive indicator for BPD.[13] In contrast, the combination of FiO2 and PEEP or mean airway pressure (MAP), simply expressed by the product of the two and referred to as respiratory severity score (RSS), has the potential to predict BPD in preterm infants.[13-15] In an explorative analysis of the PHELBI study[16], the authors could show that birth weight and RSS were related to moderate-to-severe BPD or death in extremely low birth weight infants.[13] Jung et al identified a cut-of-value 3.0 for RSS at postnatal day 14 to predict severe BPD and/or death with an odds ratio of 11.3 ($p<0.001$, 95% confidence interval, 2.8-44.6) in preterm infants with a gestational age of less than 28 weeks.[14]

Various risk factors have been identified and several predictive indicators for BPD have been proposed.[17, 18] However, models combining indicators have limited predictive value, lack extensive external validation, and are difficult to be employed in clinical practice.[17, 19-21]

A robust indicator for BPD should be easily measurable and cost-effective with high analytical sensitivity and specificity. It should predict subsequent lung disease or serve as a surrogate for long-term respiratory outcomes, provide insight into underlying pathophysiological mechanisms A validated BPD indicator has the potential to reduce healthcare costs and minimize the risks associated with the widespread use of ineffective therapies.[1]

This study aims to prospectively evaluate the predictive performance of existing and newly identified indicators for BPD. The type of predictive indicators can be classified in three categories: respiratory data including lung mechanics and lung imaging, echocardiographic data, and molecular biomarkers.[21]

4.1.1 Respiratory data

In recent years, new respiratory monitoring devices and ventilation modes have been implemented in the respiratory care of extremely preterm infants. These new technologies comprise the respiratory function monitor (RFM), lung and diaphragmatic ultrasound (LUS, DU), forced oscillation technique (FOT) for respiratory oscillometry, neurally adjusted ventilatory assist (NAVA), and, to a smaller extent, electrical impedance tomography (EIT).[22-31] All these methods give access to parameters that might have the ability to detect the development of BPD at a very early stage.

4.1.2 Echocardiographic data

BPD evolves as a result of disrupted lung alveolarization and vascularization caused by antenatal and postnatal factors. It is recognized as a multisystem disorder affecting not only the airways and lung parenchyma, but also cardiac development. The impaired gas exchange observed in BPD reflects both ventilation and perfusion abnormalities, with significant contributions from disrupted pulmonary vascular development and remodeling.[32-34] Impaired pulmonary vascular growth leads to increased pulmonary vascular resistance and elevated right ventricular pressure, contributing to pulmonary hypertension and right ventricular dysfunction.[35-43] In

addition, altered left ventricular adaptation, including diastolic dysfunction, is driven by persistent inflammation and abnormal hemodynamics, and is associated with adverse respiratory outcomes in preterm infants.[44-46]

In extremely preterm infants, assessment of these cardiac alterations is best performed using echocardiography. Targeted neonatal echocardiography (TnECHO) is recommended for early detection and monitoring of pulmonary hypertension and ventricular dysfunction in infants with BPD, particularly during the early weeks of life.[47] In the last decade, TnEcho has become a core component of neonatal hemodynamic evaluation, enabling adjudication of patent ductus arteriosus (PDA) significance, assessment of pulmonary hypertension (PHT) and ventricular performance, and early identification of circulatory phenotypes relevant to respiratory morbidity.[47-50] The American Society of Echocardiography specifically recommends TnECHO for preterm infants with persistent respiratory support or oxygen need, and for those born before 29 weeks' gestation, to screen for BPD-associated pulmonary hypertension and guide management.[47] Recent frameworks also emphasize the role of standardized echocardiographic algorithms for screening and management of chronic pulmonary hypertension in this population.[51]

4.1.3 Molecular biomarkers

Research in BPD prediction has increasingly shifted toward the identification of early molecular biomarkers.[1] Numerous studies have reported promising univariate associations between individual molecular biomarkers and the subsequent development of BPD.[20, 52, 53] However, the absence of robust validation frameworks has limited firm conclusions regarding the clinical utility of molecular biomarker prediction models.[21, 52]

In a proof-of-concept observational study, Pereira-Fantini et al. described a comprehensive plasma biomarker landscape for early BPD prediction, highlighting not only inflammatory pathways but also potential disturbances in hemostasis and tissue remodeling within the first 72 hours after preterm birth.[54] Biological markers have also been investigated in non-plasma samples, such as tracheal aspirates.[55] Collectively, recent studies underscore the potential of biological markers to predict and monitor BPD and to inform clinical strategies aimed at

protecting the critical progenitor cells and developmental processes essential for lung formation and repair.[1, 54-58]

4.2 Rationale for the Study

To assess the predictive value of emerging respiratory, hemodynamic, and molecular metrics from novel technologies for early detection of BPD in extremely preterm infants. Early identification of infants at considerable risk for developing BPD would allow for timely decision-making, individualized preventive interventions, and anticipating the evolving BPD. Because cardiorespiratory interactions play a significant role in the development of BPD, integrating TnEcho-derived hemodynamic markers may improve early risk stratification beyond respiratory metrics alone. In addition, molecular biomarkers for BPD may provide insight into the underlying pathophysiological pathways driving disease development.

4.3 Definition of BPD

We will use the following BPD severity classification, which has been shown to be highly predictive of respiratory morbidity in early childhood.[59] It is based on the need for respiratory support at 36 weeks of postmenstrual age (PMA), regardless of prior or current oxygen therapy, and defined by

- no BPD: no support.
- BPD grade 1: nasal cannula ≤ 2 L/min.
- BPD grade 2: nasal cannula > 2 L/min or noninvasive positive airway pressure.
- BPD grade 3: invasive mechanical ventilation.

4.4 The respiratory severity score (RSS)

The Respiratory Severity Score (RSS) is defined as the product of the mean airway pressure (MAP) and the fraction of inspired oxygen (FiO_2). The mean airway pressure is computed by PEEP + (PIP-PEEP) x $t_i/(t_i + t_e)$, where PIP is the peak inspiratory pressure, t_i is the inspiratory time, and t_e is the expiratory time.[60] This value is displayed by the ventilator for invasive and non-invasive ventilation modes including NAVA. For patients on high frequency oscillation ventilation, the mean airway pressure corresponds to the continuous distending pressure. For nasal continuous positive airway pressure (CPAP), the MAP value corresponds to the PEEP level. Several studies

showed that RSS is related to moderate-to-severe BPD or death in extremely low birth weight infants.[13-15]

4.5 Study outcomes

4.5.1 Primary outcomes

The primary outcomes are to:

1. assess the individual relationship between the predictive indicators (derived from LUS, DU, TnEcho, FOT, NAVA, and EIT) and RSS[13, 14];
2. prognosticate BPD at 36 weeks post-menstrual age by each predictive indicator (derived from RFM, LUS, DU, TnEcho, FOT, NAVA, and EIT).

The predictive indicators derived from RFM, LUS, DU, TnEcho, FOT, NAVA, and EIT, will be recorded at different time points as follows:

I. RFM: Peak inspiratory pressure and expiratory tidal volume (averaged over 10 highest values within each recording) will be assessed during neonatal transition and resuscitation (D0).

II. LUS and DU: The LUS score[61] and the diaphragm shortening fraction (DSF)[62, 63] will be assessed on D1, D3, D7, D14, and D28.

III. TnEcho: PDA status, PHT metrics, indices of volume overload and diastolic and systolic ventricular function as well as right and left ventricular cardiac output will be assessed on D1, D3, D7, D14 and D28, GA 32 and GA 36.[47]

IV. FOT: Respiratory system reactance (Xrs) will be assessed by FOT on D1, D3, D7 and D14 and at 3 and 5 years of age.[64]

V. NAVA: Edi min and Edi max will be assessed from a 15-minute Edi signal recording during a restful state on D3, D7 and D14.[22]

VI. EIT: Center of ventilation, overdistension and atelectasis/collapse, global inhomogeneity index, and the relationship between EELI and EELV during a single PEEP trial performed during daily routine, will be assessed on day D7 and D14, [28, 65-68]

VII. Biological samples: blood samples (50 µL) and tracheal aspirate (1 mL, only if intubated) will be collected every day on the first week of life and on D14 and D28.

where D0 refers to the first 30 min after birth (transition phase), D1 to the 12-24 hours of life, and DN (N = 3,7,14, and 28) embraces a 72-hour period from 00:00 of the $(N-1)^{th}$ day of life to 23:59 of the $(N+1)^{th}$ day of life. RSS will be assessed on D1, D3, D7, D14, and D28 during a stable and quiet respiratory state.

4.5.2 Secondary outcomes

- Uni-/multivariable logistic models of respiratory parameters, including predictive indicators of the primary endpoints.
- Mutual correlations between predictive indicators of the primary endpoints, including interstitial pneumonia pattern assessed by chest radiograph in the first week of life.[69]
- Difference of predictive indicators from LUS, DU, TnEcho, NAVA and EIT between intubated and non-intubated patients.
- Difference between longitudinal recordings (different time points) of the predictive indicators.
- Difference of measurements of LUS, DU, TnEcho, FOT, NAVA, and EIT during routinely performed PEEP/CDP trials (whenever applicable).
- Difference between measurements of LUS, DU, TnEcho, FOT, NAVA, and EIT (whenever applicable) before and after treatments (e.g., surfactant administration, surfactant lavage, postnatal steroids) that might affect lung function.
- Survival rates and major morbidities (necrotizing enterocolitis, sepsis, intraventricular hemorrhage, periventricular leukomalacia, retinopathy of prematurity).
- Univariable and multivariable logistic regression models will be used to evaluate the association between protein expression and the development of BPD.
- Neurological outcome at two and five years of corrected age which are routinely assessed during standard follow-up visits at our department.
- Respiratory outcome using FOT at 3 and 5 years of age assessed during standard follow-up visits at our department.

4.6 Study design

This study is a prospective observational cohort trial.

4.7 Study population

Preterm infants born below 26 weeks of gestation defined by time elapsed between the first day of the last menstrual period and the day of delivery[70] and admitted to the neonatal intensive care units of the Medical University of Vienna.

4.7.1 Number of subjects

For the calculation of the a-priori sample size we refer to published data for LUS and FOT:

I. Considering a 95 % power at a 5 % significance level and assuming a correlation coefficient between LUS and RSS of at least 0.6[26, 71], the a-priori sample size amounts to 98.
II. For the prediction of BPD by means of LUS, we aim for an area under curve (AUC) of at least 0.7[26]. Considering as null hypothesis the prediction by chance (AUC=0.5) and a positive/negative (BPD/no BPD) case ratio of 2 (one BPD case, two non-BPD cases), a priori sample size amounts to 93 for a 95 % power at a 5 % significant level (http://biosoft.erciyes.edu.tr/app/easyROC/).
III. Considering a logistic model with BPD as an outcome with an incidence of about 30% (unpublished data of the Medical University of Vienna in the period from 2012 to 2023) and an increase in the odds ratio for BPD of 2.0 for each increase in Xrs-z-score[72], the a-priori sample size amounts to 80 for a power of 80% at a significance level of 5% (https://sample-size.net/logistic-regression-sample-size/).

For all other indicators no preliminary data is available. Therefore, we base the a priori sample calculation on the existing data mentioned above and aim for a sample size of 100 participants who will complete the study. With a dropout rate (e.g. due to unavailable data) of around 30%, we plan to include 140 participants.

On average, 50 infants are born below 26 weeks of gestation each year at the Medical University of Vienna with a survival rate of approximately 75%. Among the survivors, the incidence of BPD (for the former definition referring to the need for oxygen > 21% at 36 weeks of PMA) amounts to approximately 30% in this cohort (unpublished data of the Medical University of Vienna in the period from 2012 to 2023).

If we assume that we will enroll around 35 extremely premature infants per year who survive up to 36 weeks PMA, the duration of the study will be around four years from the start of the study.

4.7.2 Inclusion criteria for patients

- Preterm infants born below 26 weeks of PMA (by best obstetric estimate).
- Has a parent or other legally acceptable representative capable of understanding the informed consent document and providing consent on the participant's behalf prospectively.

4.7.3 Exclusion criteria for patients

- Major congenital anomaly or anticipated alternative cause for respiratory failure.

5 Methods

5.1 Study procedures and data collection

The collection of following data consists of documenting automatically recorded data:

- **RSS parameters** – MAP and FiO2 can be read from the ventilator without interrupting the respiratory support. If the preterm infant is supported by high-flow nasal cannulas we will assume a PEEP level of 5 cmH2O.
- **RFM parameters** – All relevant parameters can be downloaded from the RFM after its use at the bedside.
- **NAVA parameters** – All relevant parameters can be downloaded from the ServoN ventilator (Maquet Critical Care AB, Solna, Sweden) without interrupting the respiratory support. We will only collect NAVA parameters if the patient is supported by a NAVA mode (invasive or non-invasive).

The collection of following data needs intervention that is part of the routine care:

- **LUS including DU** – LUS is performed regularly in preterm infants to surveil respiratory care. In this study, we plan this intervention at fixed times within a time frame of 72 hours. Thereby we make sure that routinely performed LUS can be used for this study in

most cases. In cases where we cannot perform LUS within a planned time frame, we will omit the collection of the LUS data.

- **TnEcho** – TnEcho is routinely used to evaluate circulatory status in extremely preterm infants, providing detailed assessment of PDA significance, PHT, volume status, and cardiac function. For this study, TnEcho examinations will be performed at predefined time points within a time frame of 72 hours, preferably aligned with clinically indicated scans. Examinations will be performed at the bedside without interrupting respiratory support. If a TnEcho examination cannot be performed within the planned time frame, collection of TnEcho data for that time point will be omitted.

The collection of following data needs interventions that are not part of the routine care:

- **Respiratory system reactance (Xrs)** – In the first 14 days of life, Xrs will be measured by the Forced Oscillation Technique as implemented in the Fabian ventilator (see details below). During invasive ventilation this measurement needs to be activated and takes about 3 seconds in which the patient is supported by a superimposed oscillatory signal at the same PEEP and FiO2 level. This measurement is well tolerated in preterm infants even in situations of respiratory and hemodynamic compromise.[73] During any non-invasive respiratory support, the Xrs measurement can be performed using a nasal mask and with an activated flow sensor. We will perform Xrs-measurements only if the patient is supported with the Fabian ventilator and skip the measurement if another ventilator is used. FOT measurement at 3 and 5 years of age will be performed using the CE-certified RESMON PRO FULL device (https://www.restech.it/project/resmon-pro/).[74]
- **EIT parameters** – To monitor and collect EIT parameters we need to place a textile belt (see description below) around the thorax. This can easily be done during a nursing round in a gentle way without interfering with other therapies. This is well tolerated also in extremely preterm infants. EIT data collection can be activated when the infant is in a quiet state. There is no difference in data collection between invasive and non-invasive respiratory support. After EIT measurement, the textile belt can be removed softly during the next nursing round.

- **Biological markers** – To assess plasma and tracheal protein profiles, we will collect 50 µL (approximately one drop) of blood on a dried blood spot card and 1 mL of tracheal aspirate per sample, respectively. Sample collection will occur exclusively during routine clinical blood sampling and tracheal suctioning procedures. The additional blood loss associated with study sampling is negligible. All samples will be immediately stored in a certified −80 °C freezer following collection. The samples will be processed at a later stage.

5.1.1 RFM

Respiratory function monitoring constitutes an approach to evaluate manual positive pressure ventilation quality.[75] Recordings can be used for immediate feedback and corrective steps and for subsequent analysis later. A CE-certified RFM (Neo100, Monivent AB, Gothenburg, Sweden) measures ventilation parameters (tidal volume, positive inspiratory pressure, positive end expiratory pressure, ventilation rate, mask leakage). A flow sensor needs to be placed between mask and T-piece and streams measured data in real-time to the RFM. RFM visualizes and stores data.

No changes in mask hold (e.g., ergonomics, force necessary to achieve tight seal) are required when the mask adaptor and flow sensor from the RFM are connected to the ventilation equipment. The dead air space of the mask adapter is 1.5mL, as stated by the manufacturer, and therefore, acceptable when used for manual positive pressure ventilation. The flow sensors are contained in sterile packaging, and a new flow sensor will be used for each patient.

Prior to the administration of positive pressure ventilation, the provider inserts the estimated body weight into the RFM. The RFM then displays the tidal volume already adjusted for the respective body weight (shown in in mL/kg). The RFM will be pre-installed at our neonatal resuscitation unit and will measure ventilation parameters throughout ventilation procedures. The duration of intervention will be based on the patient's clinical need.

If the subjects need positive pressure ventilation support in the postnatal transition phase, peak inspiratory pressure, expiratory tidal volume (the ten highest values within each recording will be averaged), mask leakage, and positive end expiratory pressure will be extracted from the RFM

recordings. All other respiratory parameters recorded by the RFM will be evaluated as secondary outcome parameters.

5.1.2 LUS and DU

Neonatologist performed lung ultrasound (NPLUS) is a tool for dynamic assessment of lung diseases and diseases with lung involvement and can be used for visualization during various procedures and interventions.[27, 76, 77] LUS can be used to monitor evolving lung injury and is able to predict the development of BPD in preterm infants born below 32 weeks of gestation. [78, 79]

LUS will be performed using an ultrasound imaging system with a high frequency linear transducer. It is well tolerated in extremely preterm infants and the average duration amounts to 3 minutes (SD 2 min).[71] We assess the following quantitative and qualitative LUS parameters in ventral, lateral and dorsal scans following the LUS standard: A-Lines, B-lines, size of consolidation, pleura, diaphragm movement, effusion.[80] We will use the LUS score based on classical lung ultrasound semiology to quantify the LUS findings. The LUS score is computed over 6 chest areas (3 per side) and ranges from 0 to 18.[80]

The diaphragm excursion allows interpretation of work of breathing and tidal volume.[30] Using a linear probe for the DU, the excursion is measured in subcostal transversal view orthogonal to the pleura in M-mode by measuring the difference between in- and expiration.[81] For measuring the thickness of the diaphragm, the probe is placed at the anterior and posterior axillary line between 8^{th} and 9^{th} rib orthogonal to the pleura (vertical). Two echogenic lines will be visible in the ultrasound scan: the one closer to the probe is the pleura parietalis and the deeper one is the peritoneum. The diaphragm is the hypoechogenic structure between those two lines [62, 81]. The duration of DU amounts to about 2 minutes.[62] In M-Mode, the minimal (TET) and the maximal thickness (TIT) during a breathing cycle are measured three times and mean values are considered for the diaphragmatic shortening fraction (DSF) which is determined by the following formula: $DSF(\%) = 100x(TIT-TET)/TET$.[31, 62]

LUS and DU will be routinely performed in the course of therapeutic interventions that aim at improving the respiratory conditions.

5.1.3 TnEcho

Targeted neonatal echocardiography (TnEcho) is a comprehensive bedside tool to appraise neonatal cardiovascular physiology and hemodynamics in real time. It is used to assess the hemodynamic significance of a PDA, evaluate acute and chronic PHT, and assess right and left ventricular systolic and diastolic function. TnEcho also allows differentiation between hypovolemia and impaired myocardial contractility in hemodynamically unstable preterm infants.

Examinations will be performed with a high-frequency phased-array or sector transducer at the bedside in a supine position without interrupting respiratory support. Assessed parameters include PDA status (open/closed, diameter, shunt direction, flow pattern, Iowa Score), indices of PHT (tricuspid regurgitation jet-derived right ventricular systolic pressure, interventricular septal curvature represented as eccentricity index, pulmonary vascular resistance indices derived from right ventricular ejection time and pulmonary artery acceleration time), indices of volume loading (pulmonary vein Doppler, mitral inflow E/A, isovolumetric relaxation time), qualitative ventricular function (including strain where feasible), and right and left ventricular output. Examinations will be scheduled at specific time points (D1, D3, D7, D14, D28, GA 32 and GA 36), and data from clinically indicated exams within this window will be used whenever possible.

5.1.4 Respiratory Oscillometry

Respiratory oscillometry provides respiratory reactance (Xrs) by measuring pressure and flow at the airways opening while a small-amplitude, high-frequency pressure stimulus is superimposed to ventilation. Xrs expresses the inertial and elastic properties of the respiratory system, reflecting lung volume recruitment and tissue strain. In particular, Xrs decreases as a result of lung volume derecruitment and/or lung (over) distension, and is a surrogate for the lung compliance.[82] Oscillometry has been used in very preterm infants undergoing different respiratory support modalities to assess lung function.[24, 83-87] Few studies have shown that Xrs significantly adds to clinical parameters in prognosticating respiratory outcomes in preterm infants.[64, 72, 88]

Oscillatory mechanics will be assessed with the patient in the supine position using a commercially available forced oscillation technique (FOT) that is implemented in the mechanical ventilator Fabian HFOi (FOT 150204 V5.0, Vyaire, USA) and superimposes sinusoidal pressure oscillations (amplitude 2.5 cm H2O, frequency 10 Hz) for three seconds on the ventilation waveform. For measurements performed during non-invasive respiratory support, we consider inspiratory values and compensate for leaks and the shunt of the face mask as described by Zannin et al.[64] For measurements performed during invasive ventilation, we will correct for the contribution of the tracheal tube (considering the diameter and the actual length of the tube) as proposed by Pigmans et al.[89] Xrs measurements will be routinely performed in the course of therapeutic interventions that aim at improving the respiratory conditions. Oscillometry measurements at 3 and 5 years of age will be performed using the CE-certified Resmon Pro FULL device (Restech Srl, Milano, Italy, https://www.restech.it/).[74]

5.1.5 EIT

Electrical impedance tomography (EIT) is a non-invasive method to continuously monitor changes in lung impedance which is highly correlated with gas volume.[90, 91] EIT has been successfully applied in extremely preterm infants during non-invasive and invasive mechanical ventilation to monitor gas distribution within the lungs.[92-94] We will use the neonatal EIT LuMon Belt (Sentec, Landquart, Switzerland) that is designed for preterm infants, allowing for a gentle placement around the thorax of the infant just above the nipple line and connected to the LuMonConnector (Sentec, Landquart, Switzerland) that transfers the EIT signal to the EIT monitor. For the EIT measurements, small electrical currents (3 mA, 198 kHz) will be repetitively injected in rotation through adjacent electrode pairs, and voltage changes are recorded by all passive electrodes pairs (scan rate 50 Hz). The GREIT image reconstruction algorithm as implemented in the LuMon™ System (Sentec, Landquart, Switzerland) uses this voltage data to generate a matrix of local relative impedance changes compared to a reference state.[95] EIT measurements can be performed over several hours without disturbing the patient. We will perform EIT data analysis off-line using the numeric computing platform Matlab (Version R2018, Mathworks, Natick, Massachusetts, USA). To assess gas distribution, we consider the following

parameters: center of ventilation, overdistension and atelectasis/collapse, and global inhomogeneity index that will be derived from the EIT data as described by Frerichs at al.[23]

EIT measurements will be routinely performed during therapeutic interventions that aim at improving respiratory conditions.

5.1.6 NAVA

Neurally-Adjusted Ventilatory Assist (NAVA) employs electrical diaphragmatic activity (Edi) to synchronize the ventilator with the neonate's own respiratory efforts. Spontaneous breathing is initiated by the brain's respiratory center, which sends impulses through the phrenic nerve to the diaphragm, triggering muscle contraction and facilitating air inflow through negative alveolar pressure. NAVA ventilators utilize these Edi signals to initiate and adjust ventilator support, serving as the primary trigger mechanism for breathing on the ventilator. The ventilator adjusts pressure delivery in proportion to the Edi signal and displays two forms of Edi signals: Edi_{max} for peak inspiration and Edi_{min} for tonic diaphragm activity after expiration necessary to maintain functional residual capacity.[22] The NAVA level, expressed in cmH2O/µV, is an adjustable conversion factor that translates Edi into appropriate ventilator pressure for each breathing effort. Peak inspiratory pressure (PIP) is calculated using the formula: PIP = NAVA level × (Edi_{max}–Edi_{min}) + PEEP.[22, 96] This mode aims to improve patient-ventilator synchrony and comfort by individualizing pressure delivery based on diaphragmic contraction. The mean Edi during exhalation seems to correlate with changes in PEEP levels during nasal CPAP in preterm infants.[97]

The recorded real-time data provides the opportunity to study the physiological breathing pattern as well as concomitant parameters (pressure, time of inspiration, frequency) of premature infants receiving invasive or non-invasive ventilation. We will use the software packages SERVO Tracker SCI (Version 1.0, Maquet Critical Care AB, Solna, Sweden) and Servo Analysis Tool (Version 2.0, Maquet Critical Care AB, Solna, Sweden) to obtain 10-minute respiratory data (Edi signal, pressure and flow waveforms at a sampling rate of 100 Hz) from the ServoN ventilator in real-time on a daily basis (as long as the patient has NAVA as respiratory

setting). This will be conducted bedside at the ventilator without disturbing the patient or interrupting the ventilation.

5.1.7 Molecular biomarkers/proteomics

Blood and tracheal aspirate samples will be collected and immediately stored at -80°C until proteomics analysis will be performed at a later stage. Among others, we will analyze sialic acid–binding Ig-like lectin 14 (SIGLEC-14), basal cell adhesion molecule (BCAM), and angiopoietin-like protein 3 (ANGPTL-3).[53]

5.2 Clinical data

The following clinical data will be extracted from the electronic clinical record:

- Demographics (sex, gestational age, birth weight)
- Perinatal risk factors (mode of delivery, fetal growth restriction, antenatal corticosteroids, surfactant administration)
- Age and weight at the time of the measurements
- Gas exchange and respiratory parameters at the time of measurements including respiratory support mode, mean airway pressure, fraction of inspired oxygen (FiO2), tidal volume, PEEP, PIP, respiratory rate, inspiratory time, SpO2, transcutaneous CO2, pO2, pCO2
- Laboratory tests for detecting and monitoring inflammation
- Drugs affecting lung function such as corticosteroids
- Respiratory outcomes (total duration of respiratory support, BPD classification).

5.3 Patient identification

Each enrolled patient will be given a consecutive number.

6 Analysis section

6.1 Data analysis

We will compare demographics and clinical data between infants with and without BPD using t-test (or using the Wilcoxon rank sum test if the data are not normally distributed) for continuous

variables and the Chi-square test for dichotomous variables. Correlations will be examined using Pearson's correlation (parametric data) or Spearman correlation (non-parametric data). We will use logistic regressions to assess the correlation between prognostic indicators and BPD. First, we will perform univariable regression analyses; then, we will adjust the models for gestational age and birth weight. We will investigate the ability of prognostic indicators to predict BPD using receiver operating characteristic (ROC) analysis and identify the optimal cut-off value by the Youden index method. The C-statistics will be calculated to assess the predictive performance of our models. For each prognostic indicator, we will compare the areas under the ROC curves (AUC) and apply likelihood ratio tests to determine whether the indicator significantly improves the prediction of respiratory outcomes compared to clinical factors alone. We will use Spearman correlation to evaluate mutual correlations between prognostic indicators.

In case of repeated measurements on the same subject, we will use linear mixed-effects models clustering on the individual subject to assess the difference in prognostic indicators in infants with and without BPD.

We will not correct for multiple testing due to the exploratory nature of the study.

7 Ethical section

7.1 Inconveniences and risks for subjects

All patients enrolled in this study will be continuously evaluated as implemented in the routine care at our neonatal intensive and intermediate care units. No therapeutic measures will be affected by study procedures. LUS and TnEcho are non-invasive bedside ultrasound examinations already widely integrated into routine neonatal care and will be performed during scheduled clinical assessments whenever possible, thereby minimizing additional handling and discomfort.

7.2 Risk/benefit assessment

All patients enrolled in this study receive medical care for their underlying disease. Ongoing treatment regimens will not be affected or changed by study procedures. The current study does

not imply any risk for patients involved. Findings of this study will help to better predict the respiratory outcomes of extremely preterm infants.

7.3 Informed consent

It is the responsibility of the investigator to obtain written informed consent from parents or legal guardians of each patient participating in the trial, after oral and written explanation of the aims, methods, benefits, and potential hazards of the trial. This consent must be obtained before any trial-specific procedures are performed on the patient. It will be made completely and unambiguously clear to the parents or legal guardians that they are free to refuse to participate in the trial or withdraw their consent at any time and for any reason, without incurring any penalty or withholding of treatment on the part of the investigator. Signed informed consent forms must be kept in file by the investigator and documented in the Case Report Forms.

In the delivery room it is often not possible to obtain parental consent beforehand because preterm birth is an extremely stressful situation for parents, and it is often unclear if patients will need positive pressure ventilation or other interventions after birth. Therefore, informed consent before birth would mean additional stress for parents and bias the inclusion of patients. However, it is very important to collect data in these acute situations as well, to evaluate how the quality of ventilation changes also in stressful situations and determines the further clinical course of the patient. In case positive pressure ventilations are needed, data will be recorded with the RFM and parents will be asked for consent after birth as soon as possible. If parents refuse consent, data from the respective patient will be deleted.

7.4 Ethical and legal aspects

The investigator will ensure that this trial is conducted in full conformance with the principles of the “Declarations of Helsinki”, of the European Council Convention for the protection of the rights and dignity of the human being (Oviedo, 04/04/1997), the Austrian drug law (Arzneimittelgesetz) and the Austrian act on medical devices (Medizinproduktgesetz) in the last valid version. Furthermore, it is the responsibility of the investigator to ensure that the trial is performed in accordance with the Guidelines of Good Clinical Practice of the European Commission and with the country specific regulations according to the local requirements concerning clinical studies

(ICH/CPMP/135/95 Step 4 errata), and according to the Directive (2001/20/EC) of the European Parliament and of the Council of 4 April 2001.

The trial will be submitted to the Ethics Committee for approval. If the trial takes longer than one year, an annual status report will be provided to the Ethics Committee. The end of the clinical trial will also be reported to the Ethics Committee.

7.5 Withdrawal criteria

Parents or legal guardians may withdraw their consent to participate in the trial without giving any reason. The investigator may remove the subject from the trial for any of the following reasons:

- Occurrence of exclusion criteria
- For other medical reasons

The following reasons oblige the investigator to take a patient off the trial:

- Upon parents' or legal guardians' request

Any patient removed from the trial will remain under medical supervision of the Department of Pediatrics and Adolescent Medicine, Medical University of Vienna until discharge is considered medically acceptable.

7.6 Premature termination of the trial

The trial will be terminated prematurely in any of the following cases:

- If the number of dropouts is so large that proper conclusion of the trial is no longer a realistic possibility.

Premature termination of the trial must be documented.

8 Administrative section

8.1 Documentation

The following documents must be available in the investigator`s study file prior to the enrollment of any subject:

- Copy of positive vote from the Ethics Committee;
- Sample of the informed consent form to be used;

The patients will be monitored and treated throughout the course of the trial at the Department of Pediatrics and Adolescent Medicine, Division of Neonatology, Pediatric Intensive Care and Neuropediatrics, Medical University of Vienna, Austria. Relevant findings to the study will be recorded on separate study sheets. The records will include the study number, the taking of consent, study dates of the patient, medical history or examinations, and measurements of the respiratory and cardiovascular system.

8.2 Publication of study results

Publication of study results from this investigation in an appropriate medical journal will be anticipated. At least two weeks before submission, the manuscript will be circulated to all parties involved.

8.3 Protocol amendment

If any modifications become necessary or desirable, these will be documented in writing; major changes require the approval of all investigators and the local ethics committee.

8.4 Recruitment

All patients checked for inclusion/exclusion criteria and considered at any moment for participation in the trial will be recorded on a separate list. The following data will be recorded: date of examination, unit, initials, birthday of the patient, diagnosis, major diseases, inclusion into study: yes/no, if no: reasons for non-enrollment (e.g. the exclusion criterion, participation refused, etc.).

8.5 Error correction

For error corrections in source data and study documents, the investigator or an authorized clinical staff member will cross out the part to be corrected (no correction fluid) with one line, so that the incorrect data is still readable, and then entering the correct data, initialing and dating the change.

8.6 Storage of study documents

All correspondence relating to this clinical trial should be kept in appropriate file folders. Records of subjects, source documents, drug inventory sheet and informed consent forms pertaining to the trial must be kept on the Investigator's File. Records must be retained according to guidelines governing the conduct of the trial.

According to the European Community, record storage is required for 15 years.

If an investigator leaves, withdraws, or retires the responsibility for maintaining the records may be transferred to another person who will accept responsibility. Samples will be stored in the Department of Pediatrics and Adolescent Medicine, Division of Neonatology, Pediatric Intensive Care and Neuropediatrics, Medical University of Vienna, under responsibility of the working-group leader of Neonatology.

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