Increased cytotoxic T-cells in the airways of adults with former bronchopulmonary dysplasia

Online supplement

APPENDIX 1. Detailed material and methods

Participants

a. **Study population**

This case-control study was conducted on subjects from the **LUN**g obstruction in **A**dulthood of **PRE**maturely born (LUNAPRE, www.clinicaltrials.gov/ct2/show/NCT02923648) cohort as previously described (21). Twenty-two subjects in the BPD, 22 in the preterm, 22 in the asthma group, and 24 healthy controls underwent bronchoscopy and BAL (Table E1 in Appendix 2). The current study population did not differ significantly compared to the full cohort (21). The reason for the study participants that did not complete the LUNAPRE study protocol was an unwillingness to go through bronchoscopy. All participants provided written informed consent. The study was approved by the Swedish Ethical Review Authority (ref: 201211872-31/4). The preterm born participants were recruited from a pre-existing cohort [1, 2] at the neonatal unit of Sachs' Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden, where they were admitted in the neonatal ward between 1992 and 1998. One patient in the preterm group had been admitted to the neonatal unit at Danderyd's Hospital, Stockholm. Sachs' Hospital serves as a regional neonatal centre for the Southern part of Stockholm. The former patients were contacted and received information about the study by mail and telephone calls when telephone numbers were available. The healthy controls and patients with asthma were recruited from a student website in Stockholm ("Studentkaninen",

http://www.studentkaninen.se) and through advertising on social media and in newspapers. The study was conducted between 2013 and 2017, when the participants were invited to the Department of Medicine Solna, Karolinska Institutet, Department of respiratory medicine and allergy, Karolinska University Hospital Solna, Stockholm, Sweden, and at Sachs' Children and Youth Hospital, Södersjukhuset, Stockholm. The diagnosis of BPD was based on the need for supplemental oxygen for at least 28 days and severity degree determined at 36 weeks GA according to Jobe and Bancalari [3]. The diagnosis of allergic asthma [4] was confirmed by a positive methacholine challenge test with a decrease in $FEV_1 \geq 20\%$, and sensitisation to common airborne allergens as measured by Phadiatop®. None of the participants used inhaled or oral corticosteroids, leukotriene receptor antagonists or antihistamines within three months prior to inclusion. The asthma group was not examined during the pollen season. All participants were non-smokers. Relevant data on perinatal and neonatal history was collected from the Swedish Medical Birth Registry and medical charts.

b. Lung function testing

I. Dynamic spirometry

Spirometry was measured using a Sensormedics 6200 (SensorMedics, Yorba Linda, California, USA) with the subject in the sitting position, wearing a nose-clip. All subjects performed at least three maximal expiratory flow-volume (MEFV) measurements. The highest values of forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), and maximum expiratory flow when 50% of the FVC remains to be

exhaled (FEF_{50}) were extracted and used from analysis, provided that the subject's effort was coded as being maximal by the test leader, the MEFV curve passed visual quality inspection, and that the two highest FEV_1 and FVC readings were reproducible according to ATS/ERS criteria[5]. FEV_1/FVC is expressed as a ratio. Reversibility to bronchodilator was tested using four doses of Airomir® (salbutamol) 0.1 mg/dose and repeating spirometry 15 minutes later. Reversibility was defined as an increase of 12% and 200 ml in FEV_1 according to international guidelines[5, 6].

II. Body plethysmography and diffusing capacity of the lungs for carbon monoxide (D_{LCO})

Whole Body plethysmography was performed with Vmax62 J CareFusion (SensorMedics, Yorba Linda, California, USA) to measure static lung volumes and diffusing capacity. The Vmax 62 J system is fully automatised and measures dynamic lung volumes, static lung volumes, plethysmographic calculation of FRC (Functional Residual Capacity) and diffusing capacity with single-breath method. Calculations were made from three approved SVC (slow vital capacity) breaths with a variability <150 ml and ERV (expiratory reserve volume) <150 ml. Diffusing capacity measurements were made from at least two approved figures with reproducibility <10% of the mean volume or <1.0 mmol kPa/min. Diffusing capacity was correlated with haemoglobin value (Hb) using Haemocue Hb201 DM-analyser. All calculations from the body

plethysmograph were made according to the international guidelines from ATS/ERS.

III. Methacholine challenge test

Bronchial hyper-responsiveness to methacholine was dosed utilizing a Spira nebulizer (Spira Elektro 2, Respiratory Care Centre, Hämeenlinna, Finland) according to modified protocol.[7, 8] Pre-challenge FEV_1 was measured before and after inhalation of normal saline concentration with Medicro Spirometer (Ailos Medical AB, Karlstad, Sweden). Pre FEV_1 <70% or when FEV_1 fell more than 10% post saline inhalation the subject was excluded from the test. The aerosol delivered was adjusted to 0.5 l/s seconds, with a start volume of 100 ml and a tidal volume of 0.5-1 litre. Each subject practised the nebulizer before the study. The mouthpiece was held firmly between the teeth and a nose clip was applied. Methacholine solution with concentration starting from 1 mg/ml was inhaled. Starting dose of 18μg of saline and methacholine mixture was delivered with 2 breaths with increasing dose and number of breaths every three minutes to a cumulative dose of 3520μg according to a separate protocol. Spirometry for calculation of FEV_1 was performed 2.5 min after inhalation of each dose of methacholine given. The methacholine provocation was terminated if the FEV_1 decreased with $\geq$ 20% or the maximum dose of 3520μg was given.

IV. Fractional exhaled Nitric Oxide

Fractional exhaled Nitric Oxide *(*FeNO) was measured with a chemiluminescence analyzer (EcoMedics Exhalyzer® CLD 88sp with

Denox 88, Eco Medics, Duernten, Switzerland). The procedure was performed in accordance with published guidelines.[9] The mean exhalation flow rate was 50 mL/s ± 10% during the NO plateau. The manoeuvre was repeated until two exhalations agreed to within 5% coefficient of variation (CV), or three exhalations agreed to within 10% CV. The NO concentration, FeNO, was defined as the mean of these values expressed in parts per billion (ppb). The analyser was calibrated using a standard NO calibration gas (Air Liquide Deutschland GmbH, Krefeld, Germany). In a few study subjects, FeNO was measured using a NIOX device (Aerocrine AB, Solna, Sweden) [10].

c. Peripheral blood analyses

Blood samples were obtained at an outpatient visit prior to bronchoscopy. The following analyses were carried out: leukocytes with differential counts, erythrocytes, platelets, haemoglobin, C-reactive protein, alpha-1 antitrypsin, orosmucoid, albumin, and haptoglobin. In addition, screening for IgE antibodies to a mix of common airborne allergens: birch, timothy, olive tree and mugwort, cat, dog and horse dander, mould (*Cladosporium herbarum*), and house dust mite (*Dermatophagoides pteronyssinus*) was performed (Phadiatop®, Phadia AB, Thermo Fisher). Measurements were done using routine methods at the laboratory of Karolinska University Hospital, Solna, Sweden.

d. Bronchoscopy and bronchoalveolar lavage (BAL)

Bronchoscopy and BAL were performed in the morning between 08:30 and 10:30, according to a standardised protocol [11-13]. The fasting study subjects received pre-medication with 1 ml morphine-hyoscine hydrobromide 10 + 0.4 mg/ml or morphine 10 mg/ml intramuscular and atropine 0.5 mg/ml subcutaneously to be relaxed during the procedure. Two doses of salbutamol 200 µg were inhaled before the bronchoscopy. Topical lidocaine hydrochloride-naphazoline spray 34 mg/ml + 0.17 mg/ml six to nine doses were instilled through the nose and mouth. A flexible bronchoscope (BF-P1600 or BF-P180, Olympus America Inc., Cypress, CA, USA) was inserted into the airways through the nose and additional topical lidocaine 20 mg/ml was instilled through the instrument. Also, lidocaine gel 2% was used on the instrument surface for smoother insertion. After inspection of the airways, the bronchoscope was wedged into a middle lobe bronchus and five aliquots of 50 ml each of 37˚sterile phosphate-buffered saline solution were instilled and gently suctioned back The BAL fluid was kept on ice in a measuring cup (Serres, Kauhajoki, Finland) until further processed.

I. Processing of BAL fluid

The volume of BAL fluid was measured and debris and mucus were removed by filtration (Woven Mesh Spacer, Dacron, Millipore, Merck, Darmstadt, Germany). The BAL cells were separated by centrifugation. Differential cell counts were determined on cytospin slides prepared with native pellet and stained with May-Grünwald Giemsa. In these cytospin slides, 500 cells were counted. Mast cells were stained with toluidine blue, and the number of cells within 10 visual fields (16 x magnifications) was scored and reported as the absolute number of these cells. The cell-free BAL fluid was centrifuged to eliminate cell debris and the

supernatant was stored at -80˚C until analysis. Differential cell counts were obtained in 23/24 subjects in the healthy control group, 21/22 in the asthma group, 20/22 in the preterm group, and 19/22 in the BPD group (due to technical problems and low-quality samples not useful for analyses).

II. Immunofluorescence staining and flow cytometry of BAL cells

BAL cells were washed twice with cell wash (BD, Franklin Lakes, NJ, USA) and resuspended to a concentration of 1 x 10^6 cells/100µL. After surface staining and additional washes, BAL cells were fixated and permeabilized (BD Transcription factor buffer set Cat. No. 562574) for 40 minutes at 4 ºC in the dark, washed twice before flow cytometric analysis. For intracellular staining, the cells were stained intracellularly with anti-FOXP3 (clone 259D/C7- PE-CF594, #562421BD Biosciences) for 25 minutes at 4 ºC. Finally, the cells were washed, first with permeabilization buffer and next with cell wash. Three different panels of monoclonal antibodies were used to characterize the major lymphocyte subsets and the T cell differentiation subsets in BAL, see Table E2 in the online data supplement. BAL cells were stained with surface markers. Lymphocyte subsets in BAL were analysed using flow cytometry (FACS Fortessa; BD Biosciences), and were processed in FACSDiva 6.1.2 (BD Biosciences) and analysed using FlowJo 10.7.1 (BD Biosciences). Three different panels of monoclonal antibodies were used to describe the major lymphocyte subsets and the T cell differentiation (Table E2). Flow cytometric data were excluded from the data analysis if fewer than 50 events were detected in the final gate. Flow cytometric of BAL lymphocytes was completed successfully in 17/24 of the subjects in the healthy control, 22/22 in the asthma, 21/22 in the preterm, and 19/22 in the BPD group,

respectively (due to technical problems and low-quality samples not useful for analyses).

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III. Enzyme-Linked Immunosorbent Assay (ELISA) of BAL fluid

Protein concentrations of Granzyme B and perforin in BAL fluid were quantified using commercial ELISA kits, including Human Granzyme B DuoSet (R&D Systems) and Human Perforin ELISA Set (Diaclone), respectively, in accordance with the manufacturer's protocol. Before analysis, BAL fluid was 10X concentrated using Amicon® Ultra centrifugal filters (10,000 MVCO, Merck Millipore). The final levels of granzyme B and perforin in each sample were determined by extrapolating OD values against standard concentrations using the standard curve and normalising with the correction factor for each sample. The assay range for granzyme B was 39.1 - 2500 pg/ml, whereas for perforin 62.5 pg/ml - 2000 pg/ml with a level of detection (LOD) of 40 pg/ml. Levels of granzyme B and perforin that were below the indicated assay range or LOD, respectively, were plotted as a half amount of the lower assay range or LOD (19.6 pg/ml – granzyme B; 20 pg/ml – perforin).

e. Statistical methods

Demographical data are presented as median and range for continuous variables, or numbers and percentages for categorical variables. Comparisons between the groups were performed using the Kruskal-Wallis test with *post hoc* analysis using Mann-Whitney U-test. Pearson's χ-squared and Fisher's exact test was used when appropriate. BAL lymphocyte group comparisons were performed using Mann-Whitney U-test with correction for multiple hypothesis testing using false discovery

rate (FDR) according to Benjamini-Hochberg. Statistical relevance was determined as $P < 0.05$ and FDR < 0.30 [14, 15]. The FDR level was selected to limit type I error rates while optimising statistical power. As the use of p-values alone is limiting in determining relevant alterations, effect sizes are also reported [16, 17]. The effect size was calculated using package rstatix function wilcox_effsize() from Z statistic divided by the square root of the total amount of samples. Effect size ranges from 0 to almost 1 with 0.1-0.3 representing small effect, 0.3-0.5 representing moderate effect and more than 0.5 representing large effect. Correlations were assessed with Spearman's test. Analyses were performed with Stata 13.1 (StataCorp LP, College Station, TX, USA), or in R 4.0.3. We found no significant correlations between different T-cell subsets and perinatal data in both preterm groups such as days with supplemental oxygen, days on a ventilator, days with CPAP, birth weight, or gestational age (data not shown).

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