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Analysis of neonatal immunity: innate lymphoid cells in cord blood and oxidative stress in exhaled breath condensate of children born preterm

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Project 2 – Parma (Italy)

Oxidative stress and ADMA in exhaled breath condensate of intubated preterm children: preliminary data page 67

Project 1 – Imperial College London, Inflammation Repair and Development

Analysis of neonatal immunity: innate lymphoid cells in cord blood

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Abstract

Recent studies in mice and humans suggest that asthma is not just an adaptive IgE-mediated allergic inflammatory disease driven by T helper lymphocytes. There is considerable data proposing that the pathogenesis also involves a novel cell population of the innate immune system called type 2 innate lymphoid cells (ILC2s). These cells do not express the surface markers of T lymphocytes, but have the capacity to produce IL-4, IL-5 and IL-13. ILC2s are induced by the innate epithelial cytokines IL-33, IL-25 and TSLP and are considered important in initiating allergic type 2 immune responses and contribute to the persistent airway inflammation associated with severe asthma.

Our laboratory has recently found that children with severe asthma and continuous symptoms despite high doses of corticosteroids have a greater number of ILC2s in bronchoalveolar lavage, sputum and blood compared to non-asthmatic children. It is proposed that persistent symptoms and eosinophilic inflammation in severe asthma that is relatively resistant to steroid therapy is mediated by ILC2s. We therefore aimed to culture a purified population of these cells to study their functional responses in vitro to innate epithelial cytokines with and without steroids.

ILCs were quantified in tonsils and cord blood by means of flow cytometry. The proportion of ILCs identified in tonsils was very low and inadequate for culture. For this reason the experiments focused on ILCs isolated from cord blood. We showed: 1) a higher percentage of ILC2s in boys suggesting that ILC2s can be the reason why boys tend to have more atopic disease from birth until puberty; 2) ILC2s are capable of producing more IL-13 than CD4+ T cells and can be implicated in the Th2 response seen in pregnancy. When cord blood ILCs were cultured with different pro-inflammatory mediators with or without budesonide preliminary results suggest steroid sensitivity of ILCs.

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Background

Innate lymphoid cells

Innate lymphoid cells (ILCs), a new family of lymphoid hematopoietic cells of the innate immune system, have been subject of intense research during the past few years. These are emerging as important effector cells of inflammation and homeostasis at mucosal surfaces in multiple tissues and inflammatory diseases [1] [2, 3]. ILCs represent approximately 0.01% to 0.1% of circulating lymphoid cells [1]. They originate from a common precursor (Id2 and gamma chain IL-2 receptor positive) [4], have a lymphoid morphology but are not antigen specific, lack specific T cell receptors and their surface does not express the common molecules such as CD3, CD4, CD19 that identify lymphocytes. Therefore, they are non-T, non-B lymphoid cells and are defined as lineage negative (Lin-) cells [2].

Since ILC effector function and transcription factor requirement partially resemble those of T lymphocytes, they are considered the innate form of lymphocytes [5]. ILCs are often divided into two main lineages: killer ILCs and helper-like ILCs that mirror CD8+ cytotoxic T cells and CD4+ T helper cells, respectively [6]. Killer ILCs are IL-7Rα^{-} and represent the conventional natural killer (NK) cells, which resemble cytotoxic CD8+ T cells in some aspects because they display cytolytic activity and produce cytokines, primarily IFNγ. NK cells, mediate host defence against both tumor and virus-infected cells, express Eomesodermin (Eomes) and transcription factor T-bet, required for their development and function. Helper-like ILCs express IL-7Rα (CD127) and depending on transcription factors, extracellular markers and cytokine production are further classified into group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s) [7]. ILC1s express transcription factor T-bet and secrete IFNγ and have a role in responses against protozoa and intracellular bacteria. ILC2s express the transcription factor GATA binding protein 3 (GATA3), produce primarily the cytokines IL-13 and IL-5, they act against helminths and are involved in allergic responses [8] [3, 9, 10] [11, 12]. Furthermore, ILC2s have been shown to play an important role in wound healing, tissue repair and tissue fibrosis [13]. ILC3s express the retinoic acid receptor related orphan receptor (RORγt) and produce IL-17 and IL-22. While relatively rare in the systemic circulation in comparison to other hematopoietic cells, ILCs are enriched at epithelial barrier and mucosal surfaces and act as regulators of chronic inflammation and tissue remodelling, linking innate and adaptive immune responses.

ILC groups and parallels between innate and adaptive immune lineages are shown in Figure 1.

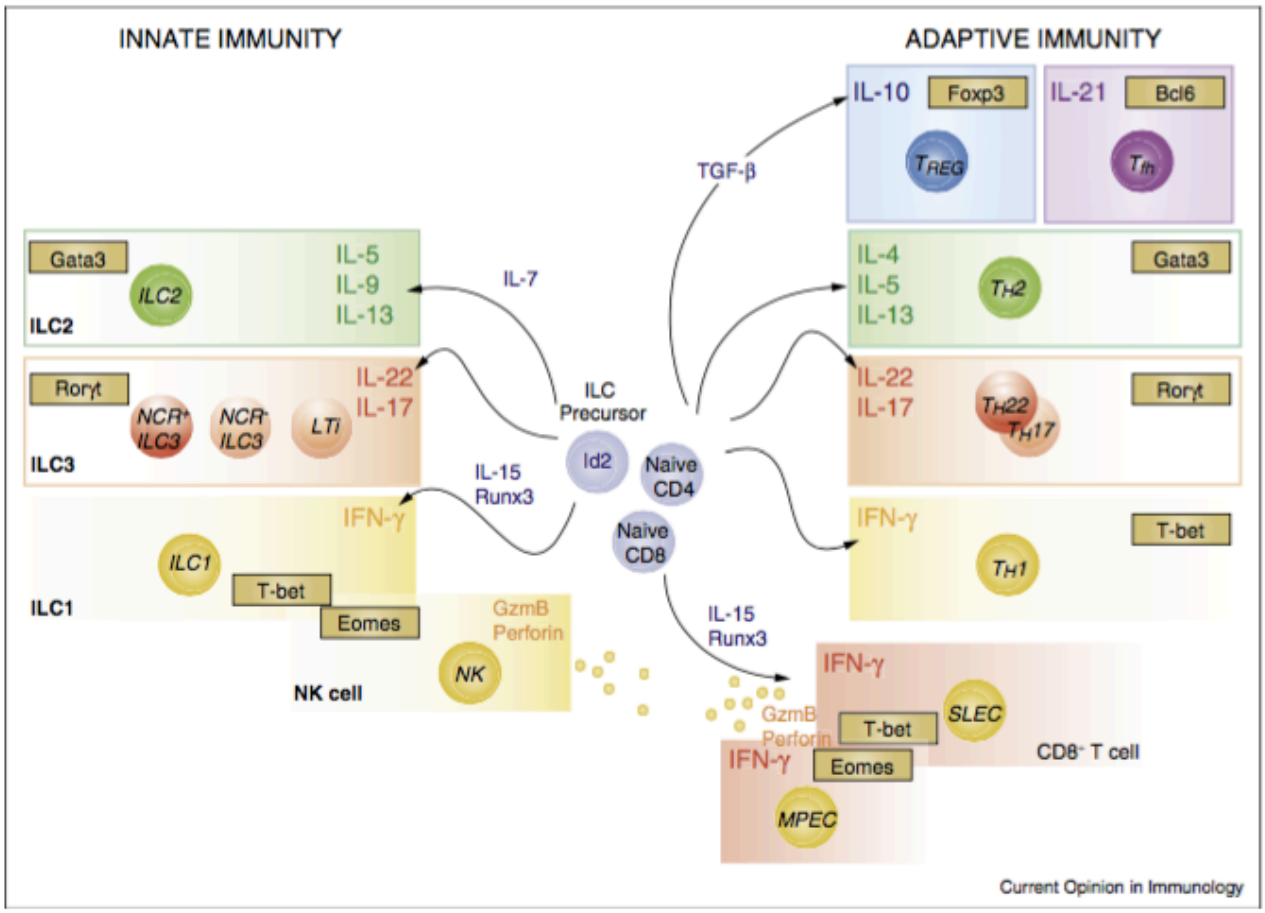


Figure 1. Innate and adaptive immunity cells [14].

Allergic asthma and ILC2s

Asthma is traditionally thought to be a disease driven by the adaptive immune system via CD4+ Th2 cells. The Th2 cytokines IL-4, IL-5, IL-13 are thought to play an important role in the induction and pathogenesis of asthma following sensitization to environmental allergens [15, 16]. IL-4 induces the production of IgE by B cells [17], IL-5 is crucial for activation of eosinophils and their migration into the lung [18] and IL-13 is associated with airway hyper responsiveness, mucus hyper secretion and airway remodelling [19]. Immunologically asthma is characterized by eosinophilic inflammation, airway hyperresponsiveness and structural airway changes, collectively termed airway remodelling [20]. Clinically asthma manifests with episodic or persistent symptoms and signs of airway obstruction which either reverse spontaneously or with medication.

About one third of all infants and preschool children develop wheezing with 30% of them developing asthma in the following years. To date, the cause of preschool wheeze and factors determining progression to asthma is unclear and better definition of the pulmonary immune environment of early life may be

essential to understand asthma pathogenesis.

Although the majority of children with asthma achieve good control with low-moderate doses of maintenance inhaled steroid therapy, approximately 5% remain poorly controlled. Children with asthma presenting with frequent exacerbations and/or persistent symptoms despite high dose of inhaled steroid treatment are classified under the term “problematic severe asthma” [21]. This umbrella term includes difficult asthma and severe therapy resistant asthma (STRA). Patients with difficult asthma have potentially modifiable factors including poor medication adherence, poor inhaler technique and continued exposure to allergens or co morbidities like obesity. They account for more than 50% of cases referred to the difficult asthma team at specialized asthma centres like Royal Brompton Hospital, London (UK) [22]. The minority of patients that are refractory to maximal treatment despite optimizing management including ensuring adherence to medications are defined as having STRA [23].

The pathology of severe asthma in children involves predominantly eosinophilic airway inflammation and atopy [20]. However, asthma is a heterogeneous disease with several clinical and pathological phenotypes [24] and there is evidence that not all patients fit the description of a Th2 mediated disease [25]. In particular, children with STRA have airway remodelling and eosinophilia in the absence of significantly elevated levels of bronchial Th2 cytokines [20]. The fact that they remain symptomatic despite high doses of steroids suggest that other molecular pathways rather than Th2 cells may be mediating the disease. An altered response of the innate immune system may be implicated in airway inflammation, altered tissue repair and remodelling. Although type 2 cytokines were almost absent in airways of severe asthmatic children, our group found an elevated number of cells positive for IL-33 in their bronchial submucosa [26]. This expression was associated with increased thickness of the reticular basement membrane, suggesting IL-33 may promote airway remodelling [27].

IL- 33 is an innate cytokine derived from the airway epithelium in response to different stimuli (pollution, allergens, infections, tobacco smoke…) [28] and has been associated with the initiation and potentiation of allergic asthma [29]. IL-33 was the first cytokine demonstrated to activate murine ILC2s and to induce a robust type 2 immune response independent of adaptive immunity [9]. The IL-33 receptor, named ST2, is present on ILC2s but also on eosinophils, macrophages, dendritic cells, basophils, mast cells and NK T cells [18]. In a neonatal murine model of inhaled house dust mite exposure IL-33 remained elevated in allergen challenged mice despite steroid administration suggesting that IL-33 may be steroid resistant [30].

Airway epithelium is considered to play a crucial role in the pathogenesis of severe asthma: apart from acting as an effective structural barrier, with IL-33 it also secretes other innate cytokines, including IL-25 and thymic stromal lymphopoietin (TSLP). IL-25, TLSP and IL-33 in particular are crucial for ILC2

development and activation in response to allergen [31] and viral triggers [32-35] inducing them to produce IL-4, IL-5 and IL-13 without the need of the development of an adaptive immune response [9] [36] [37] [38].

Considered as the innate equivalent of Th2 cells, ILC2s produce large amounts of type 2 cytokines even in the absence of adaptive immunological responses [39], induce the recruitment and activation of eosinophils and mast cells and promotes mucus secretion, and airway hyperreactivity (Fig. 2). This new reservoir of type-2 cytokines is thought to be involved in acute pulmonary allergic inflammation as suggested by numerous murine studies of allergic airways disease [15]. Allergens, like house dust mite (HDM) or *Alternaria alternata* have been reported to stimulate lung ILC2s via the release of IL-33 with consequent airway inflammation, eosinophilic infiltration and mucus overproduction [8, 10, 40].

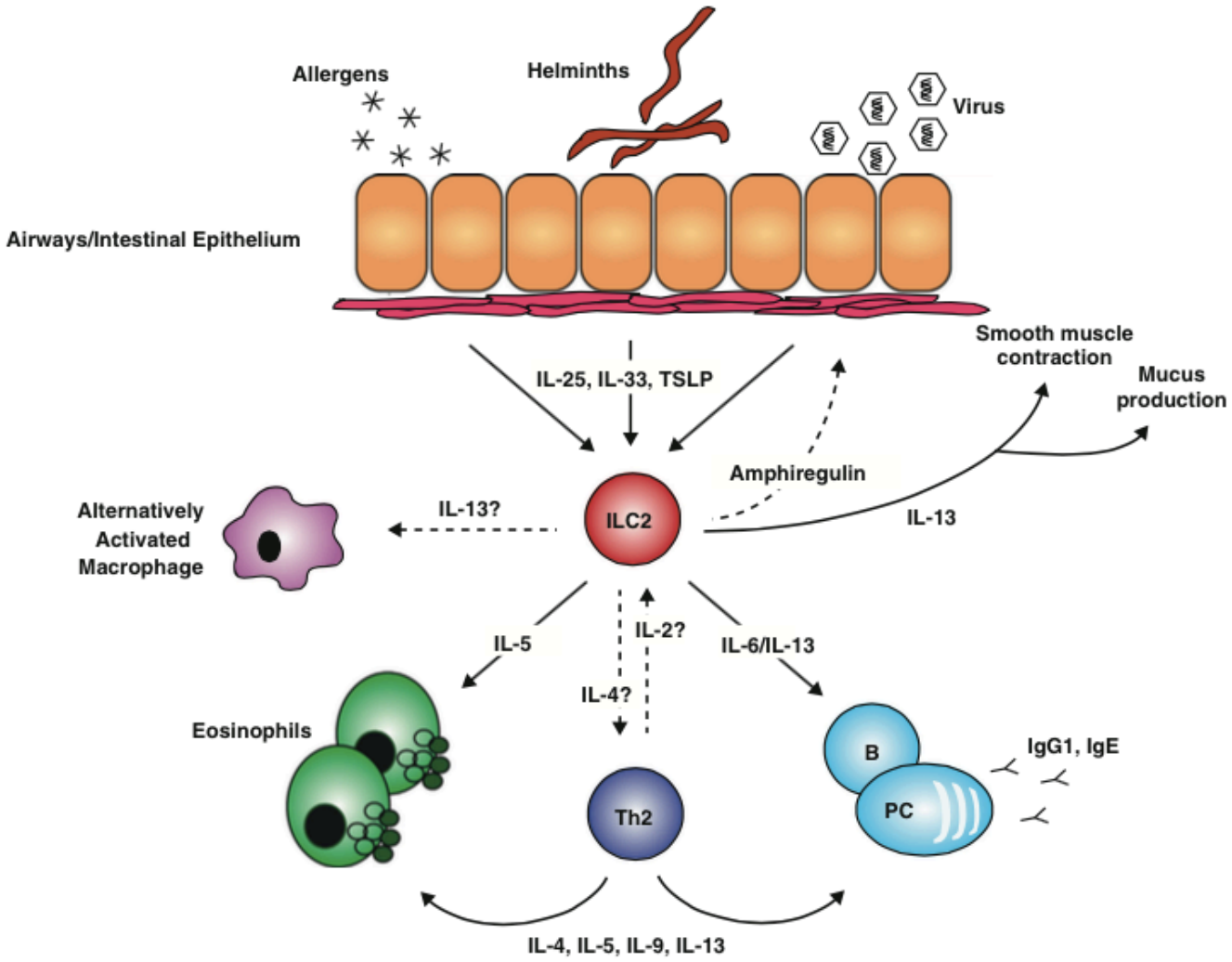


Figure 2. Innate epithelial cytokine, ILC2 and Th2 cell interactions in response to allergen and virus [15].

In humans ILC2s are estimated to be less than 0.1% of all CD45+ cells in tissues and have been found in blood [41] [42] [43], sputum [44] and broncho-alveolar lavage (BAL) [45] from adult asthmatics where they seem to correlate with airway eosinophilia [43] and asthma severity [46]. They have also been found in lung parenchyma [42] and BAL of lung transplanted patients [47], in patients with idiopathic pulmonary fibrosis [48] (34), in nasal polyps and tonsils of patients with chronic rhinosinusitis [1] [49] and in cord blood [50].

In STRA children our group found for the first time increased levels of ILC2s (defined as CD45+Lin-CRTH"+ cells) in sputum, BAL and blood [51]. Based on this finding and on the previous studies on IL-33, we therefore hypothesized that ILC2s induced by IL-33 may mediate severe asthma and may be resistant to steroid treatment thus driving airway inflammation even in steroid treated patients. The aim of the current study was generating cultures of ILC2s to better understand the functional role that these cells have in atopy and corticosteroid resistance and assess their potential as therapeutic targets. We decided to look for ILCs in tonsils of atopic and non-atopic children, considering their richness in lymphoid cells, and in cord blood, on the hypothesis suggested by Forsberg et al. that cord blood ILC2s might have greater potential of producing cytokines [50].

Hypothesis

Innate epithelial cytokine and ILC interactions mediate steroid resistance in response to allergen in children regardless of atopic status.

Objectives

1. To establish a protocol of isolating and culturing T lymphocytes and ILCs from tonsils of atopic and non-atopic children undergoing clinically indicated tonsillectomy for obstructive sleep apnoea (OSA) and/or recurrent infections
   - to find a gating strategy on flow cytometry to identify and isolate ILCs and specifically ILC2s from tonsils
   - to separate CD45+Lin- cells and specifically ILCs from tonsils by means of magnetic separation
   - to culture CD45+Lin- cells and specifically ILCs from tonsils
2. To establish a protocol of isolating and culturing CD45+Lin- cells and specifically ILC2s (Lin-CD45+CD127+CRTH2+) from cord blood
   - to find a gating strategy on flow cytometry to isolate ILCs sub-types (ILC1s, ILC2s and ILC3s) from cord blood
   - to culture isolated CD45+Lin- cells and specifically ILCs from cord blood

Aims

1. To define the lymphoid populations (CD4+, CD45+Lin- cells and ILC sub-types) in tonsillar tissue obtained from children undergoing clinically indicated tonsillectomy for OSA and/or recurrent infections;
2. To define the lymphoid populations (CD4+, CD45+Lin- cells and ILC sub-types) in cord blood;
3. To assess the interactions between the innate epithelial cytokine IL-33 and sorted CD4+ lymphocytes and ILCs in response to steroids *in vitro.*

Study design

Prospective study to assess the interactions between the innate epithelial cytokine IL-33, ILC2s and CD4+ cells in response to corticosteroids.

Methods

Tonsils were used to define characteristics of ILCs as they are a well-known source of lymphocytes and are tissues of the upper airway always in contact with inhalant allergens. Cord blood was used because it is the optimal and earliest sample that represents the "naïve" neonatal immune system, and is also a rich source of PBMCs. Furthermore, functional characteristics of ILCs had not been investigated in either a tissue source or peripheral source from children at the time of this study.

1) Tissue collection

Tonsils were collected after clinically indicated tonsillectomy performed either for recurrent infections or obstructive sleep apnoea syndrome (OSAS) from children aged 11 SD:3.9 at Chelsea & Westminster Hospital (London). Children with OSAS were referred from the Royal Brompton Paediatric Sleep and Ventilation Unit. Clinical history for wheezing, allergies or asthma was ascertained by questionnaire on the day of surgery. Blood tests including full blood count, specific IgE (RASTs) for the common inhaled allergens (list the allergens) and total IgE was obtained under general anaesthetic for surgery.
Umbilical cord blood was obtained from term babies delivered by elective caesarian section at Chelsea & Westminster Hospital (London). Maternal medical history for allergies, maternal peripheral blood eosinophil count (when) and baby gender and birth weight were recorded.
Parental informed consent to use tonsil tissue and cord blood for research was obtained either on the day of the operation or from the mother on the day of the delivery. Ethical approval was already in place for the use of human samples.

2) Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs isolation from tonsils
Extracted tonsils were transferred on ice from operating theatre to laboratories in medium (RPMI 1640 medium containing L-glutamine 300 mg/L, Gibco - Life technologies) complete of 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml) and 10% fetal calf serum (FCS) formally known as complete medium. The tissue was washed in sterile water, weighed, manually dissected with a sterile scalpel tip and eventually filtered through a 70 micron cell strainer grinding the tissue through the sieve with a syringe plunger (50). A total cell count was undertaken using the white cell counting fluid (phosphate-buffered

saline (PBS), 4% acetic acid and 2% of 1% crystal violet in methanol) and a haemocytometer. The required number of cells then progressed to processing.

PBMCs isolation from cord blood

Cord blood was transferred from the theatre to the laboratories at room temperature into Ethylenediaminetetraacetic acid (EDTA) bottles and processed within 4 hours. Total amount (ml) of cord blood was recorded and then diluted 1:1 with PBS in a 50 ml conical tube. The diluted blood was filtered through a 70 micron strain to remove potential clots and then layered on a density gradient medium (LymphoPrep, Stemcell technologies) (15 ml of LymphoPrep for a maximum of 30 ml of diluted blood) and centrifuged at 1800 RPM for 30 minutes at room temperature without brakes. Granulocytes and erythrocytes have a higher density than mononuclear cells and they therefore sediment through the LymphoPrep layer during centrifugation whereas mononuclear cells (lymphocytes, monocytes and thrombocytes) remain in the interphase (Fig. 3). The interphase layer was aspirated with a disposable pipette and diluted with PBS to fill a 50 ml conical tube and then centrifuged at 1200 RPM for 10 minutes at room temperature. The resulting pellet was resuspended with 5 ml of red blood cell lysis buffer (eBioscience) and left at room temperature for 10 minutes. To stop lysis reaction PBS was added after 10 minutes to the cell suspension and the solution centrifuged at 1200 RPM for 10 minutes at room temperature. The resulting pellet was reconstituted in complete RPMI and cells counted using white cell counting fluid.

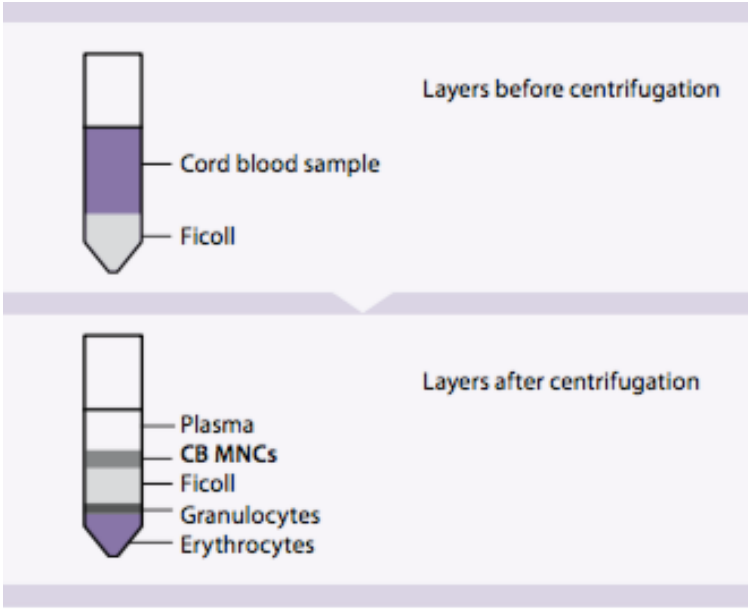


Figure 3. Schematic figure of a density gradient centrifugation (from Miltenyi Biotec protocol).

3) Cryopreservation of cord blood PBMCs

After cell counting the solution containing PBMCs was centrifuged for 10 minutes at 1200 RPM at 4°C. Pellet was resuspended with cold cryofluid (90% FCS + 10% Dimethylsulfoxide, DMSO) to obtain a maximum concentration of 10 x 10^6 cells/ml. Cryovials (1.2 ml) were filled with 1 ml aliquotes of this solution and stored for a minimum of 2 hours to a maximum of 12 hours in the vapour phase above the liquid nitrogen of a dewar or into a cryo-freezing container (Mr. Frosty, NALGENE labware) to freeze the cells slowly by reducing the temperature at approximately 1°C per minute. Then, cryovials were stored in liquid nitrogen.

4) Thawing of Cryopreserved Cells

Depending on the required number of cells to be thawed, cryovials were removed from liquid nitrogen, placed in a 37°C water bath and shacked for a few minutes. The vials were taken from the water bath when there was still a small piece of ice in the suspension and placed in the hood. Warm complete RPMI was slowly added to cell suspension which was centrifuged at 1200 RPM for 10 min at room temperature. Once removed the supernatant, cells were resuspended in warm complete RPMI and placed in the incubator for 2 hours. After 2 hours cells were counted and check for viability with 0.4% Tryptan blue exclusion.

5) Cytospin

Cord blood PBMCs were evaluated by cytospin before and after freezing. 50 000 cells were suspended in 100 uL of PBS and placed in appropriate sample chambers assembled with filter paper, labelled glass slides and the metal cassette in the cytospin 3 cytocentrifuge (Thermo Scientific Shandon, USA) and spinned at 1400 RPM for 4 minutes. Slides were dehydrated in 100% methanol for 10 minutes and after 2 hours rehydrated in A+B solution (50 ml solution A (9.47g/l NaH_2PO_4 in demineralised H_2O) + 50 ml solution B (13.609g KH_2PO_4 in demineralised H_2O) + 900 ml demineralised H_2O) for 30 min. Slides were then stained with Wright Giemsa (Sigma-Aldrich, USA) for 7 minutes. This step was followed by 7 minutes back in A+B solution. Slides were then washed and left to air dry. Slides were covered with cover slips attached with surgipath (Leica Biosystems, Germany).

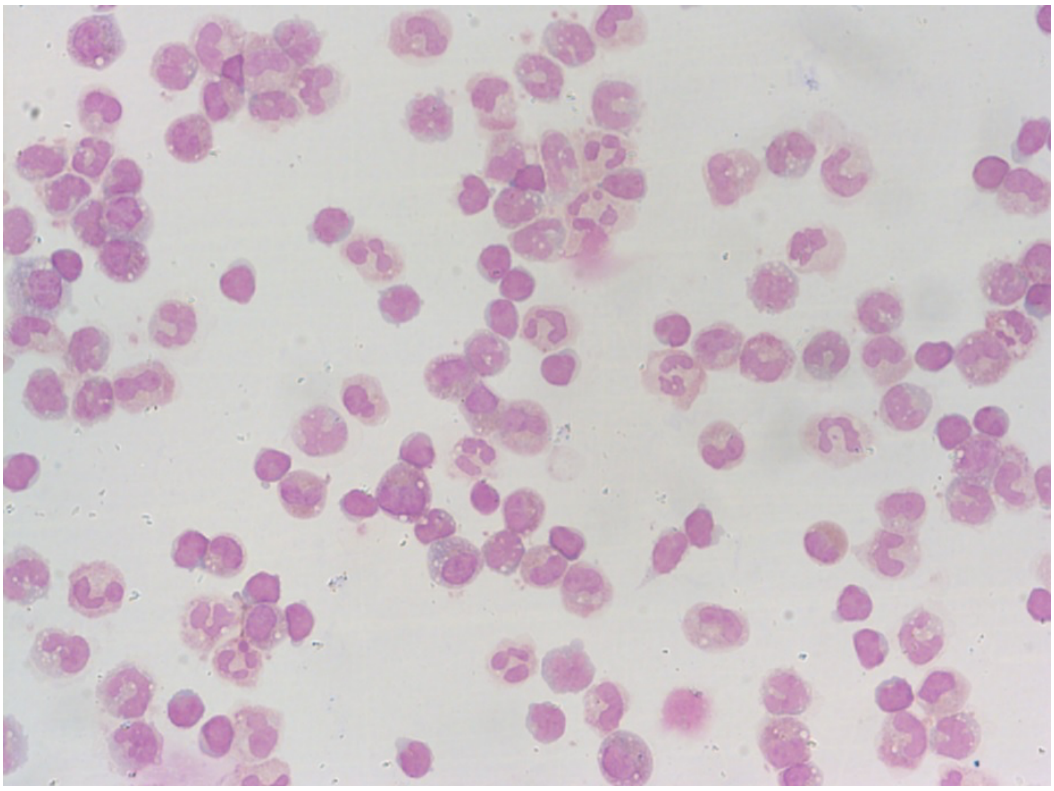


Figure 4. Example of a cytospin slide derived from cord blood.

6) Epithelial culture from tonsil brushing

The luminal surface of tonsils was scraped with a brush usually used for bronchial brush during bronchoscopy and cells gently dislodged with a pipette into a conical tube with 5 ml of Bronchial Epithelial Cell Growth Medium (BEGM). The tube was then centrifuged and the pellet resuspended with 5 ml of BEGM medium and transferred in a T-25 flask. The brush was placed in another flask with 5 ml of the same medium. Every other day the medium was changed and the flask checked for epithelial growth.

7) Identification of Lin- cells and ILC phenotype in tonsils and cord blood by flow cytometry

Flow cytometry is a laser-based technology where a fluid suspension of cells is analysed by electronic detectors. Cells are stained with antibodies attached to different fluorochromes that, excited by the lasers, emit a specific wavelenght collected by the detectors. Cell morphology and granularity, cell surface (CD markers), intranuclear (transcription factors) and intracellular antigens (such as interleukins) can be evaluated.

Freshly isolated tonsil-extracted cells and fresh or thawed cord blood-derived mononuclear cells were plated at a concentration of 1 x 10^6 cells/well and at 5 x 10^5 cells/well respectively in 96 round bottom plates and stimulated for 3 hours in a humidified incubator with phorbol 12-myristate 13-acetate (PMA) and Ionomycin in combination with Brefeldin to prevent cytokine release from the cells. After the stimulation time cells were prepared for flow cytometry according to the laboratory protocol to phenotype cell populations and assess the proportion of CD3+ cells and ILCs.

Laboratory protocol included staining with primary conjugated antibodies (all from Biolegend or eBioscience) for cell viability, extracellular markers, intracellular cytokines and intranuclear transcription factors.

Different buffers were used according to the steps of the staining protocol: FACS buffer (PBS, 1% bovine serum albumin, 1mM EDTA 0.5 M, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - Hepes 1 M) for extracellular staining, Permeabilization and FoxP3 buffer (both from eBiolegend) for intracellular staining and cell fixation respectively.

We used an anti-human Lineage (Lin) cocktail containing different antibodies against the common clusters of differentiation (CD) present on different cells but not ILCs to differentiate between a Lin positive (Lin+) and a Lin negative (Lin-) population. ILCs could be then identified in the Lin- fraction and expressed CD45, CD161 and CD127

For this purpose we used both a pre-made and a home-made Lin- cocktail. The pre-made Lin- cocktail (Biolegend) (clones are described within parentheses) included the anti-human CD3 (UCHT1), CD14 (HCD14), CD19 (HIB19), CD20 (2H7) and CD56 (HCD56) and was used on tonsils and on the first 6 samples of cord blood.

The home-made Lin- cocktail was used on cord blood PBMCs and was produced to obtain better results in terms of selecting ILC2s. This cocktail included the following anti-human antibodies (clone name within parentheses): anti-CD1a (HI149) for antigen presenting cells, anti-CD3 (OKT3), CD5 (L17F12) and CD8 (SK1) for T cells, anti-CD11c (3.9) for dendritic cells, monocytes and macrophages, anti-CD14 (63D3) for monocytes/macrophages and neutrophils, anti-CD16 (B73.1) for NK cells, activated monocytes/macrophages and neutrophils, anti-CD19 (HIB19) and CD20 (2H7) for B cells, anti-CD34 (581) for hematopoietic progenitors, anti-FCεR1a (AER-37) and anti-CD123 (6H6) for basophils and mast cells (all from BioLegend). All antibodies were properly titrated to optimize the panel, identify the correct concentration of the antibody and determine the best signal-to-noise ratio.

8) PBMCs culture

Once extracted as previously described, PMBCs from tonsils and cord blood were placed in a T-25 culture flask at a concentration of 2 million/ml in complete medium supplemented with 1% Hepes 0.5 M, 1% essential aminoacids, 1% sodium pyruvate and 1% glutamine and left overnight in a humidified incubator at 37°C and 5% CO_2 to allow monocytes to be adherent to the flask surface and be separated from the lymphocytes that instead remain in suspension. The day after all of the medium was removed from the flask, transferred to a 50 mL conical tube and centrifuged at 1200 RPM for 10 minutes at room temperature. The pellet was re-suspended in fresh supplemented medium, the cells counted and transferred to 24 or 48 well plates at a concentration of 1 million/well or to a T-25 flask at a concentration of 2 million/ml. Different stimuli were added to the culture: IL-2 (20 or 100 UI/ml), IL-7 (20 or 50 UI/ml) and IL-33 (50 UI/ml) were added in different combinations both to tonsil and cord blood lymphocytes, house dust mite (HDM) (30 ug/ml) or Tuberculin PPD (2 T.U./0.1 ml, Denmark) 6 ng/ml to tonsil lymphocytes if the subject was classified as atopic or non-atopic according to blood examinations, and budesonide (10^{-7} or 10^{-8} M/L) to cord blood lymphocytes. IL-2 and IL-7 promote ILC2 homeostasis and proliferation through the receptors IL-7R and IL-2R respectively; both IL-2 and IL-7, when associated with IL-33, increase IL-13 production from ILC2s and ILC2 activation in vitro [48] [52] [10].
A defined number of wells or flasks were left in supplemented complete medium only as controls.
Every second day, half of the medium was replaced and, in the determined conditions, ILs supplemented.
On day 3, 5 and 7 cells were harvested, counted with 0.4% Tryptan blue exclusion and stained for flow cytometry according to the protocol. The supernatant was collected.

9) Magnetic separation

The magnetic separation (MACS, Miltenyi) allows obtaining pure populations from a suspension of cells through their labelling with MACS MicroBeads, 50-nm superparamagnetic particles that are conjugated to highly specific antibodies against a particular antigen on the cell surface. The unlabelled cells pass through a column placed in a magnetic field and while the magnetically labelled cells (enriched population) are retained within the column, the unlabelled cell fraction (depleted population) flow-through and can be collected. Magnetic separation was attempted to obtain a population of lymphocytes depleted of CD3+ and CD19+ cells and assess whether or not the resulting fraction was adequate to initiate ILCs cultures.

MACS separation of tonsil PBMCs

In tonsils total mononuclear cells yields range from 5×10^8 to 5×10^9 cells per pair of tonsils. B cells comprise 60% to 70% of the cell population, while 30% to 40% are T cells and 1% to 8% are monocytes (Watanabe et al., 1974). Dendritic cells comprise <1% of the cell population (Johnston A 2009, Hart and McKenzie, 1988).

Since the main populations in tonsils are B (CD19+) and T (CD3+) cells we performed the magnetic depletion with anti-CD19 and anti-CD3 MicroBeads. Lymphocyte suspension was labelled with anti-CD19 and anti-CD3 MicroBeads and loaded into the MACS column placed in the magnetic field. The magnetically labelled CD19 and CD3 cells were retained within the column (CD19+CD3+ enriched population) whereas the unlabelled cells (CD19-CD3- depleted population) run through.

MACS separation of cord blood PBMCs

A lineage negative kit containing several antibodies against the most common CD (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, CD235a) (MACS, Miltenyi) was used to isolate the Lin- cells from cord blood. The standard technique described above was applied.

To assess the efficacy of the magnetic separation, a fraction of the enriched and the depleted population were stained and examined by means of flow cytometry.

The remaining cells were resuspended in complete medium and cultured in 24 well plates at a concentration of 1 million/well as previously described.

10) Sorting of ILCs from cord blood PBMCs using flow cytometry

To obtain a pure population of ILCs and initiate the functional experiments, cord blood PBMCs were prepared for Fluorescence-Activated Cell Sorting (FACS) (FACSaria, BD). When dealing with frozen cells, a minimum number of 25 x 10^6 cells were thawed out and stained with specific extracellular markers in order to recognize and sort a population of CD45+Lin-CD161+CD56- cells and a population of CD4+ cells. The resulting sorted cells were resuspended in supplemented medium, left overnight in a humidified incubator in a 96 well plates (at a concentration of about 10.000 cells/well) and stimulated the day after with IL-2 (100 UI/ml), IL-7 (50 UI/ml) and IL-33 (50 UI/ml). Cells were harvested at day 1, 3, 5 or 7 depending on the experiments and analysed by flow cytometry. The flow cytometry panel used for the sorting is shown in Fig. 5.

			Sorting
355	**UV 450/50**	**Live/Dead**	
405	Violet 450/50	BV421 (PB)	Dapi
	Violet 525/50	BV505	
	Violet 610/20	BV605	CD45
	Violet 710/50	BV711	
488	Blue 530/30	FITC	LIN-
	Blue 695/40	PerCP5.5	
561	Yellow 585/15	PE	
	Yellow 610/20	PE Texas Red	CD56
	Yellow 780/60	PECy7	CD161
640	Red 670/14	APC	
	Red 730/45	Alexa700	CD4
	Red 780/60	APC-Cy7	

Figure 5. Flow cytometry panel for the sorting of Lin-CD45+CD161+CD56- cells and CD4+ cells from cord blood PBMCs.

Results

Experiments on tonsils

Tonsil cellularity

Four families of patients undergoing tonsillectomy consented to tissue donation, 1 with proven atopy, none with proven OSAS. Mean age of patients was 11.6 yrs SD:3.9 yrs and mean tonsil weight 3.75 g SD:0.72 g. Median number of lymphocytes/g of tissue was 384.56 x 10^6 (248.35-800 x 10^6).

The flow cytometry gating strategy used to identify B, T and NK cells is displayed in Fig. 6 whereas the strategy used to identify ILC2s is shown in Fig. 7.

A first flow cytometry panel including the pre-made Lin- cocktail was used to describe ILC2s in tonsils (UV 350/50 Live/dead dye, BV421 IL4, BV505 IL17, BV605 CD3, FITC Lin- cocktail, PerCP5.5 CD127, PeTexasRed CD45, PeCy7 CD161+, APC IL13, AlexaFluor647 GATA3, APCCy7 CRTH2).

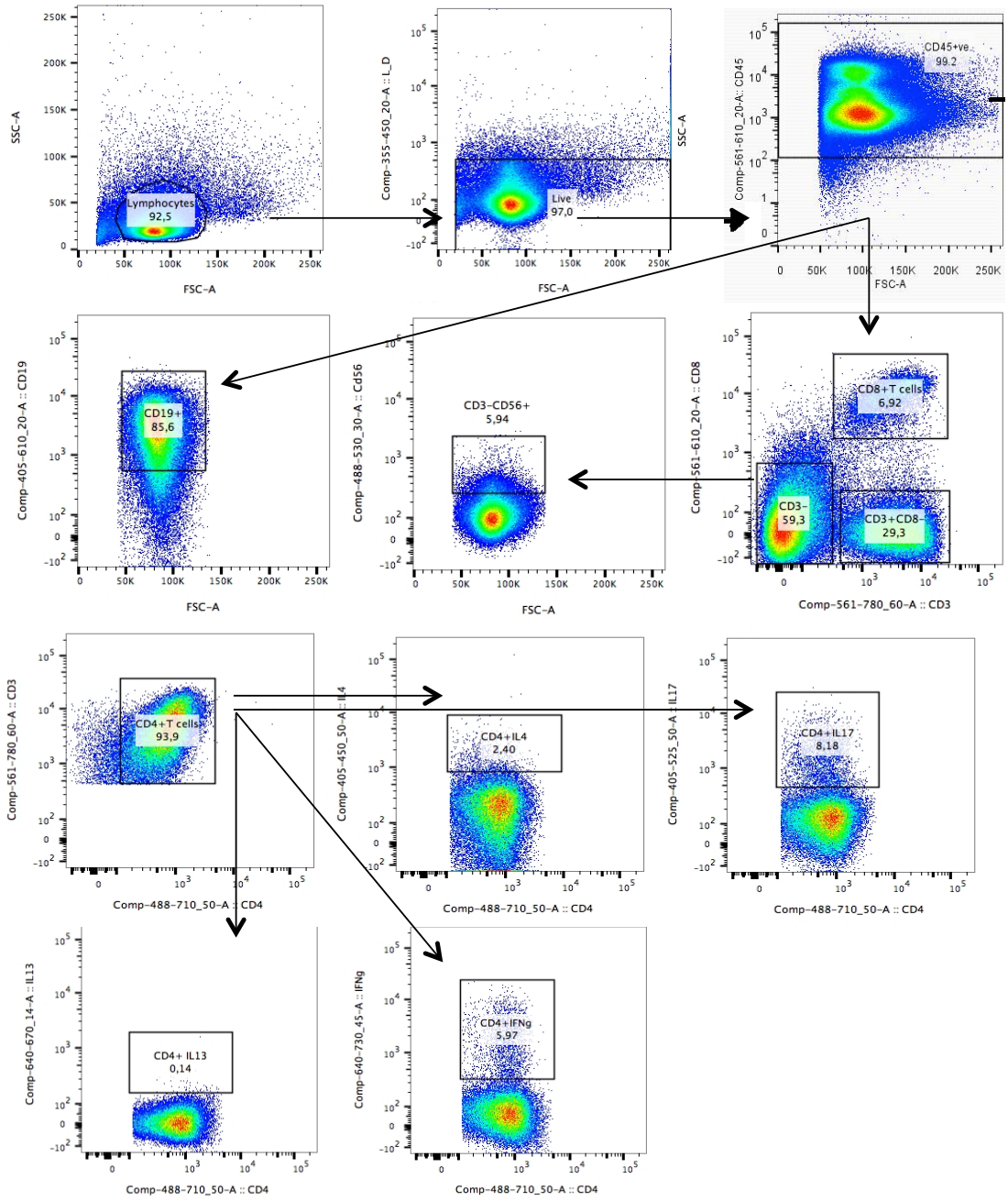


Figure 6. Gating strategy for T, B and NK cells. Lymphocytes were initially identified by size and granularity (FSC-SSC), then gated for alive and CD45+ cells. The following gating strategy was used to identify B (CD19+), T (CD3+CD4+ or CD3+CD8+) and NK cells (CD3-CD56+). CD3+CD4+ cells were described depending on their positivity for intracellular interleukins (IL-4, IL-17, IL-13, IFNg).

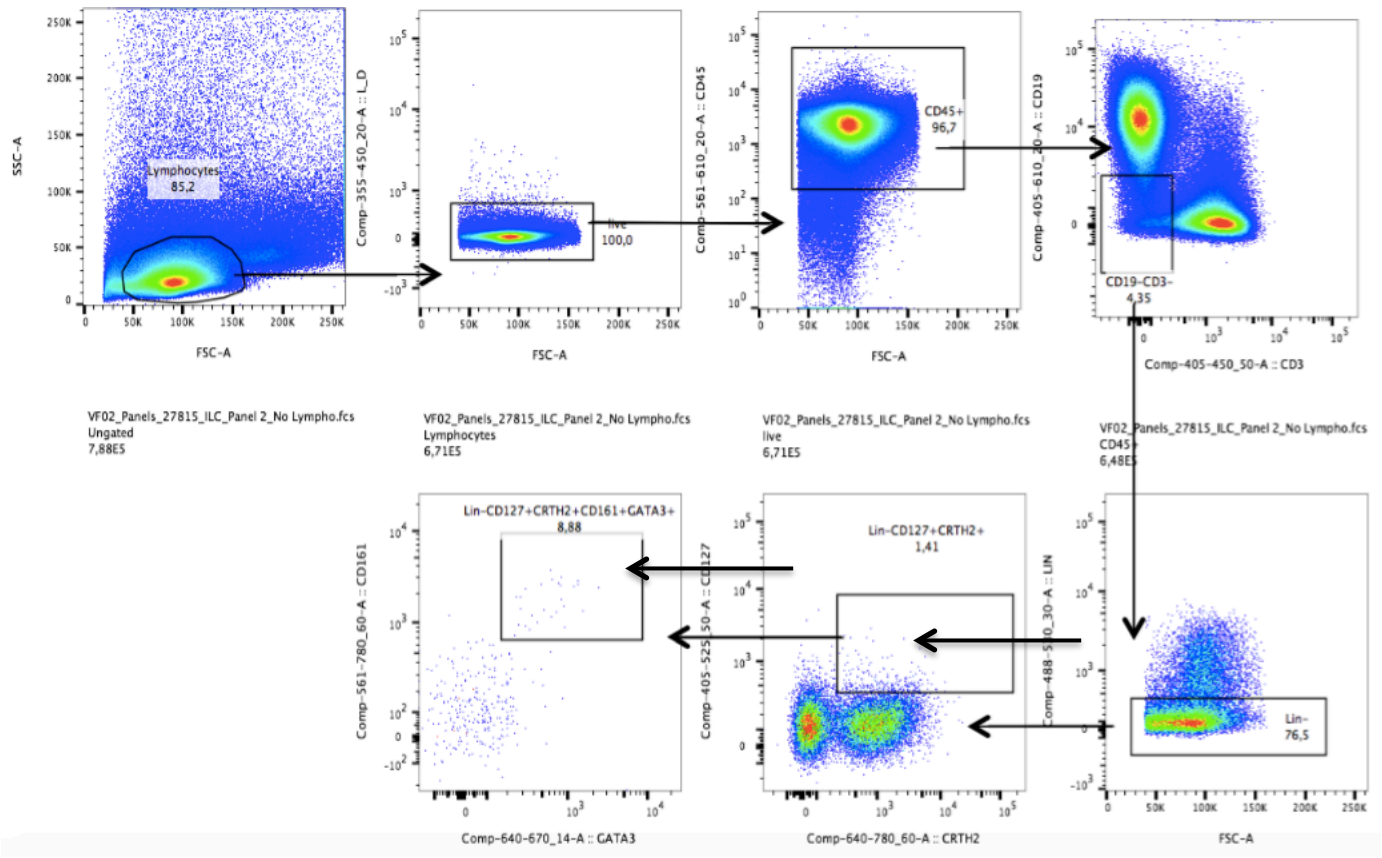


Figure 7. Gating strategy for ILC2s.

According to the initial FSC-SSC lymphoid gate, alive lymphocytes were gated for CD45+ followed by the exclusion of CD19+ and CD3+ cells. The Lin- gate was applied on the remaining cells that were then gated for CD127+ and CRTH2+ followed by CD161+ and GATA3+. The resulting population of CD45+Lin-CD127+CRTH2+CD161+GATA3+ cells were identified as ILC2s.

B cells (CD19+) and T cells (CD3+) were the most prevalent cell populations (56.4% SD:8.3% and 33.1% SD:6.8% of alive lymphocytes). Described as percentage of live CD45+ lymphoid cells, the mean values of CD45+Lin- cells and CD45+Lin-CD127+CRTH2+ cells were 0.55% SD: 0.31% and 0.0045% SD:0.0064 respectively.

Culture of tonsil lymphocytes

Previous cultures of tonsil PBMCs in common complete medium performed by other colleagues in the laboratory had given disappointing results in terms of viability (data not shown). To assess the viability of tonsil PBMCs in a different medium, the first culture experiment was done with 30 x 10^6 fresh PBMCs

placed in a T-25 flask at a concentration of 1.5 million/ml in a complete medium supplemented with 1% Hepes 0.5 M, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate.
Every second day half of the medium was changed and the cells counted. On day 3 and then every 3 days human IL-2 was added at a concentration of 20 ng/mL. Cell counting showed a small increase in cell number on day 6 (35.5 x 10^6) followed by a progressive decline until day 15 (15 x 10^6) when cells were harvested and stained for flow cytometry. On flow cytometry cell viability was 24.4%, most of the population was constituted by T cells (T cells 96.9%, B cells 1%) and the CD45+Lin-CD127+CRTH2+GATA3+ fraction was 0.033% of the alive CD45+ lymphoid cells (absolute number of cells: n= 85).
Having seen that the supplemented medium gave more stability to the culture, we chose to use this medium for the future cultures.
In order to assess lymphocyte response to different stimuli, PBMCs from different tonsillar samples were placed in 48 well plates at a concentration of 1 million cells/well with the supplemented medium. All experiments included overnight resting. On day 1, 3 and 5 half of the medium was changed and IL-2 (100 UI/ml), IL-7 (20 UI/ml) and IL-33 (50 UI/ml) were added to the culture in different combinations (i.e. medium only, IL-2+IL-7, IL-2+IL-7+IL-33). A certain number of cells were analysed with flow cytometry on day 3, 5 and 7. No significant difference in viability (Fig. 8) or cell proportions was noted in the different culture conditions.

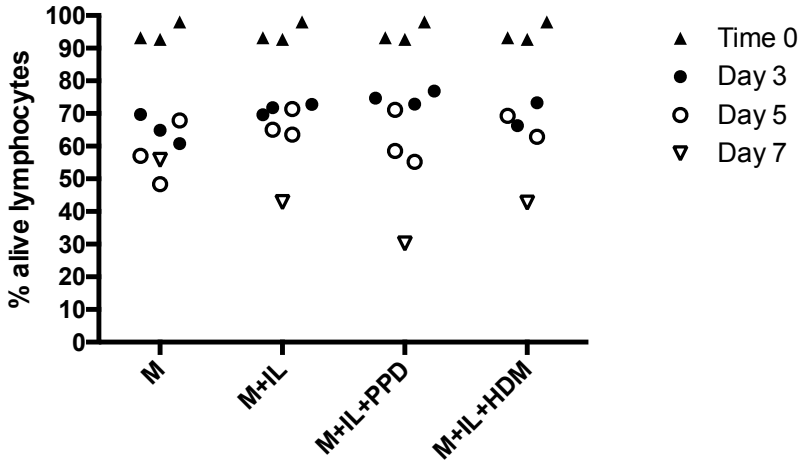


Figure 8. Results from the culture of tonsil lymphocytes. The graph shows the percentage (%) of viable lymphocytes in different conditions: M, complete supplemented medium; M+IL, supplemented complete medium + IL-2, IL-7, IL-33; M+IL+PPD, supplemented complete medium + IL-2, IL-7, IL33 + tuberculin,

M+IL+HDM, supplemented complete medium + IL-2, IL-7, IL-33 + house dust mite) along the 7 days culture.

Supplemented complete medium: RPMI 1640 medium containing L-glutamine 300 mg/L, 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml) and 10% fetal calf serum (FCS) supplemented with 1% Hepes 0.5 M, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate.

Cytokines were added to the culture with the following concentrations: IL-2, 100 UI/ml; IL-7, 20 UI/ml; IL-33, 50 UI/ml.

During the culture alive CD45+Lin- cells progressively decreased and their cytokine production did not show any significant difference in the 4 conditions tested (data not shown).

Epithelial culture from tonsil brush

The first brushing of the luminal surface of the tonsil showed cell growth and cell confluency around 70-80% on day 13 (Fig. 9). Cells were harvested on day 20, spun down and counted (total cell counting: 500.000 cells). Cells were then splitted in a chamber slide 8 well (15.000/well), kept in the incubator overnight to favour their attachment to the slide and stimulated for 6 hours with HDM and Alternaria (30 ng/ml). After 6 hours the supernatant was frozen and the cells left with BEGM medium for 48 hours until a further overnight stimulation with the same stimuli. After the second stimulation supernatants were collected and frozen and cells fixed and stained for immunoistochemistry with the monoclonal mouse IgG1 for cytokeratin 19. TLSP was measured by ELISA in supernatants with negative results (Fig. 10).

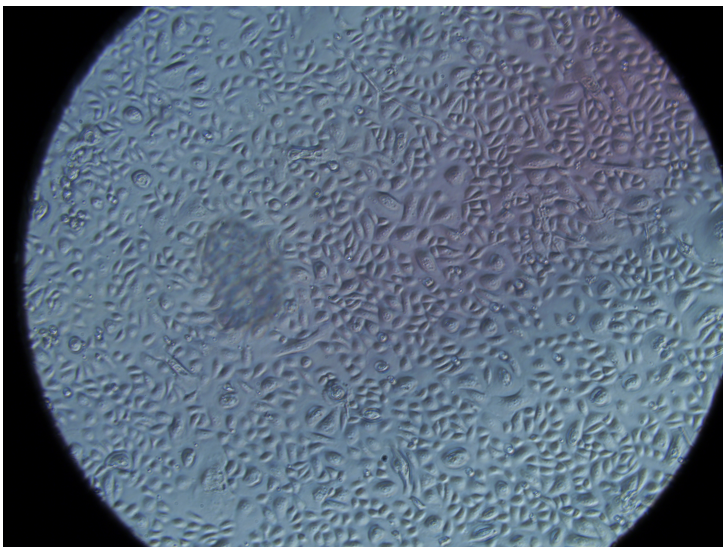


Figure 9. Microscope image (40x) of confluent epithelial cells derived from tonsil brush.

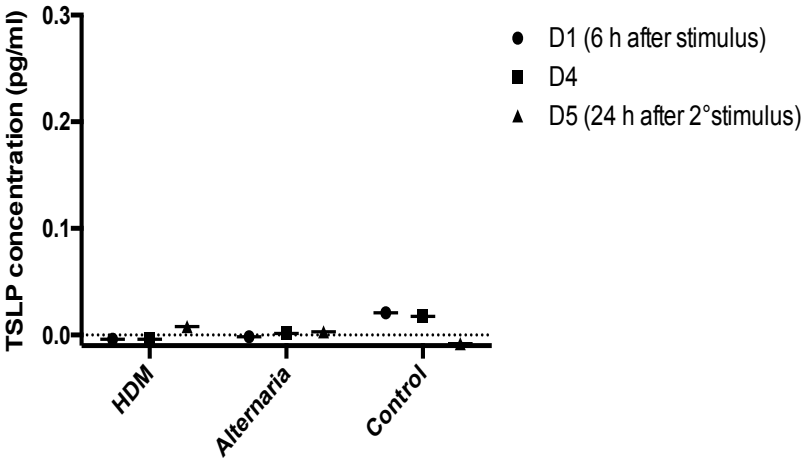


Figure 10. ELISA results of TSLP concentration in supernatants of epithelial cell culture stimulated with HDM and Alternaria at different time points (Day 1, Day 4 and Day 5) VS control.

The epithelial culture was attempted 3 times with good results in the first experiment only. Given the inconsistent results, this assay was abandoned.

Magnetic separation of CD19+ and CD3+ cells from tonsil PBMCs

Magnetic separation on tonsil PBMCs was undertaken to reduce the presence of B and T cells and increase the proportion of Lin- cells to start a culture of ILCs.

On average 100 million fresh PBMCs were coated with anti-CD19+ and anti-CD3+ beads and loaded into the magnetic column. Depending on the experiments, the resulting depleted population (CD19-CD3-) ranged between 1.7 and 25.3% of the initial number of cells. Cell lost varied between 12.2 and 81.7%. Figure 11 shows the results of magnetic separation on 4 different samples of tonsil PBMCs.

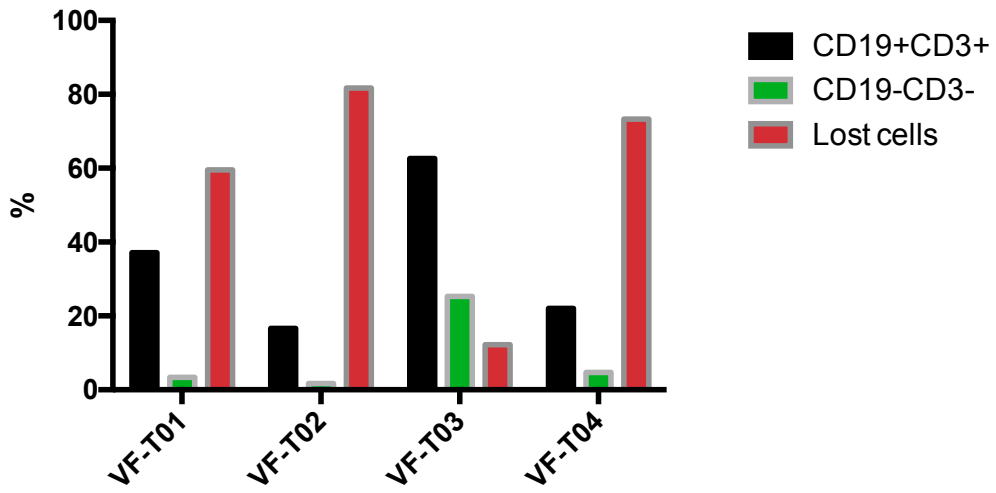
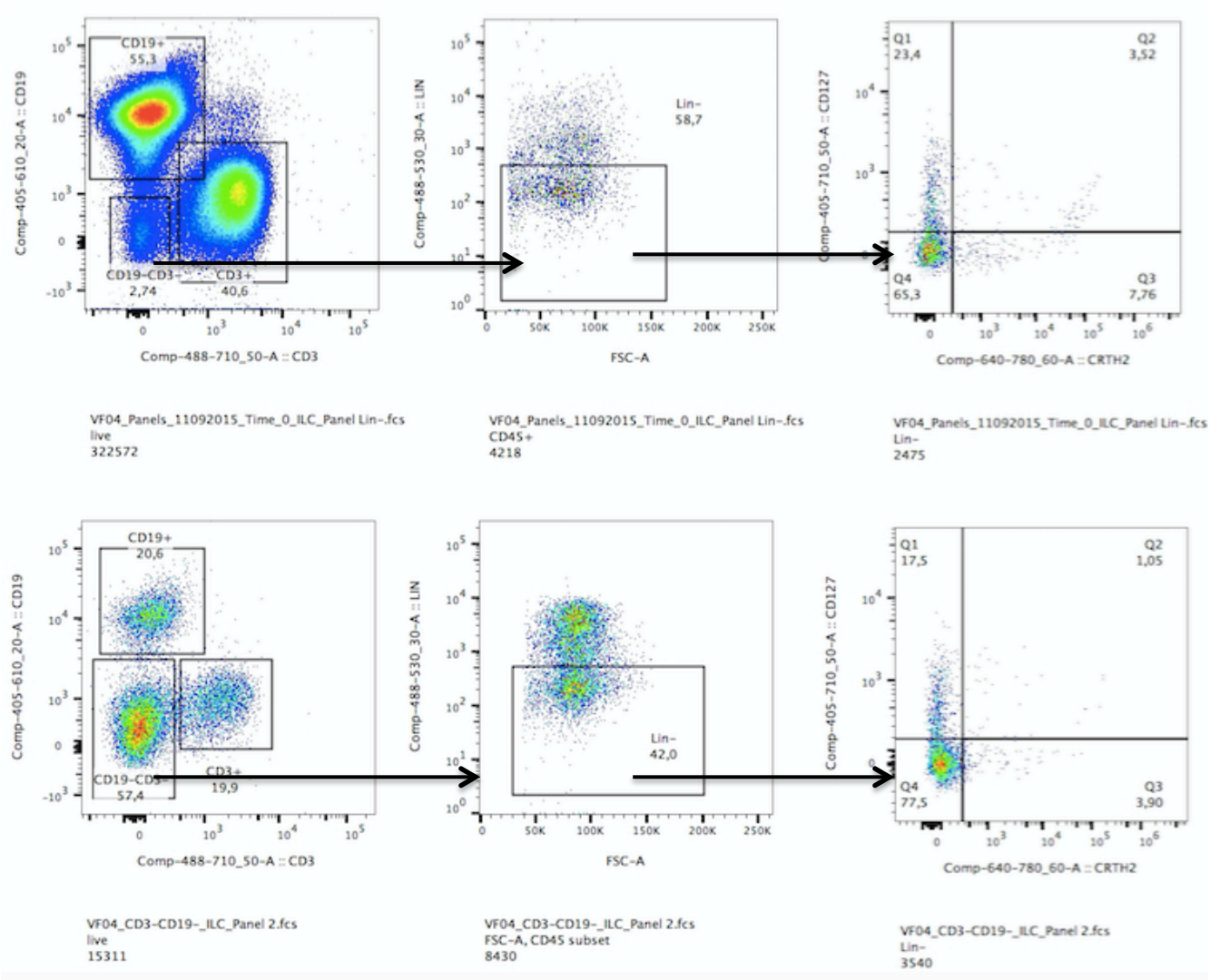


Figure 11. Results from MACS separation of tonsil lymphocytes. Percentages of CD19+CD3+ (black bar), CD19-CD3- cells (green bars) and related cell lost (red bars) obtained after magnetic separation of fresh PBMCs with anti-CD19 and anti-CD3 beads in 4 patients named VF-T 01, 02, 03 and 04.

The efficacy of magnetic separation was assessed by counting the PBMCs before and after the separation and analysing the unseparated sample and the resulting enriched (CD19+CD3+) and depleted (CD19-CD3-) populations by flow cytometry. Compared to the initial cell proportions seen in the unseparated sample, magnetic separation allowed obtaining a final cell population with a greater percentage of CD19-CD3- cells. Flow cytometry plots comparing cell populations before and after the separation are shown in Fig. 12.

A



B

Figure 12. Flow cytometry plots showing the proportions of T (CD3+) and B (CD19+) cells, Lin- fraction and CD45+Lin-CD127+CRTH2+ cells in the unseparated sample of fresh PBMCs (A) and in the same sample after magnetic separation with anti-CD19 and anti-CD3 beads (B).

Flow cytometry analysis after the magnetic separation of the 4 samples depleted of B and T cells is shown in Fig. 13.

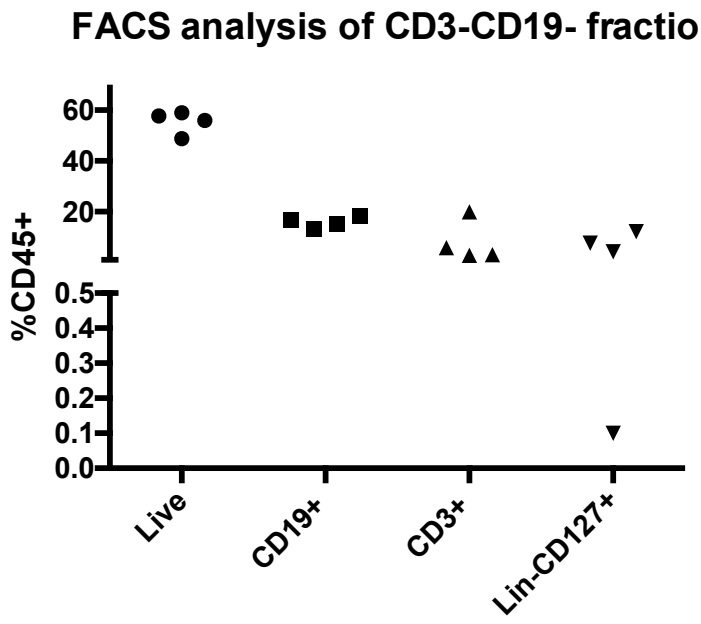
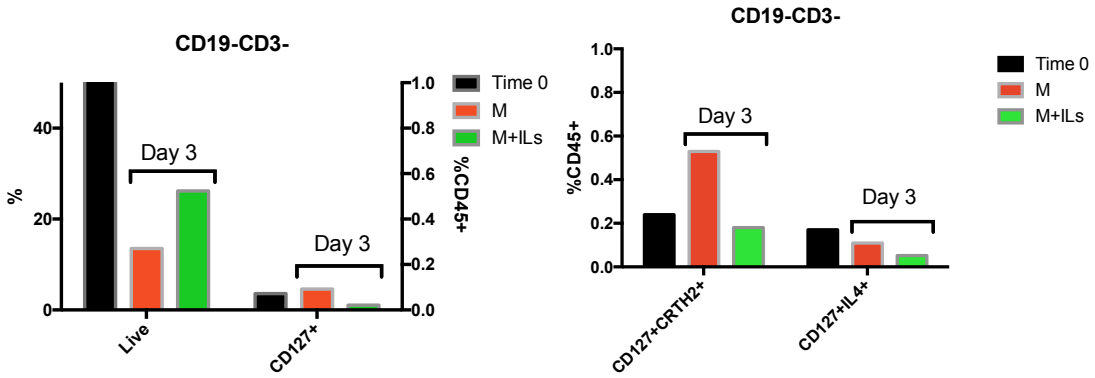


Figure 13. Cell proportions in the depleted population (CD45+CD19-CD3-) of 4 samples after magnetic separation with anti-CD19 and anti-CD3 beads.

The depleted population CD19-CD3- was cultured for 3 days with supplemented medium only or with supplemented medium and IL-2 (100 UI/ml), IL-7 (20 UI/ml) and IL-33 (50 UI/ml) (added 24 hours after the separation). The CD19-CD3- fraction showed low cell viability at day 3 (<30%) and ILC2s (CD45+Lin-CD127+CRTH2+) decreased despite the presence of the interleukins. Parallel cultures with the unseparated samples of PBMCs were done to assess whether ILCs survived and grew more or less together with CD3+ cells and no significant difference was noted (Fig. 14).

A)



B)

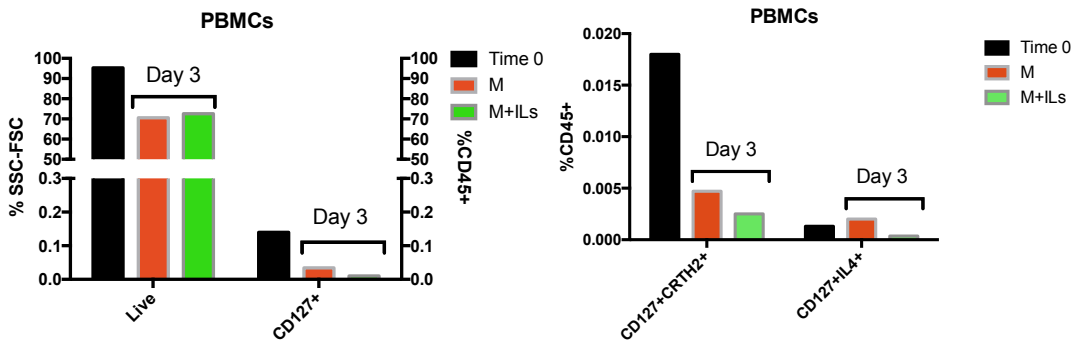


Figure 14. Results of a 3-day culture of the: A) depleted population CD19-CD3-; B) unseparated sample of PBMCs. M: supplemented complete medium, M+ ILs: supplemented complete medium + IL-2, IL-7, IL-33.

Supplemented complete medium: RPMI 1640 medium containing L-glutamine 300 mg/L, 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml) and 10% fetal calf serum (FCS) supplemented with 1% Hepes 0.5 M, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate.

Cytokines were added to the culture 24 h after separation with the following concentrations: IL-2, 100 UI/ml; IL-7, 20 UI/ml; IL-33, 50 UI/ml.

Experiments on cord blood

Six cord blood samples (3 M, 3 F) (mean quantity 9.1 ml SD:2.8 ml, median number of PBMCs 8.2 million/ml, 1-17) were collected and analysed with flow cytometry to compare the proportion of ILCs with tonsils. The same Lin- cocktail (including CD3, CD14, CD19, CD20 and CD56) and the same flow cytometry panels described for tonsil were used.

Compared to tonsils, cord blood showed greater proportions of CD45+Lin- cells and ILC2s (Fig. 15).

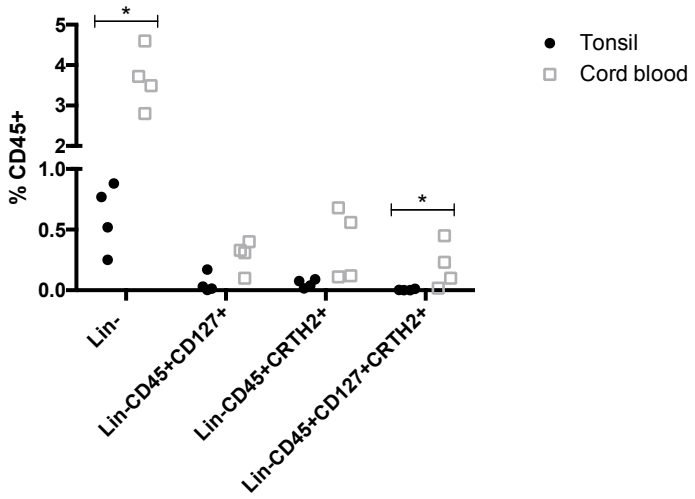


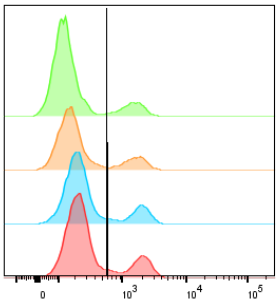
Figure 15. Proportions of CD45+Lin- cells in tonsil and cord blood. * $p < 0.05$.

To phenotype cord blood for ILCs, we processed for flow cytometry both fresh and frozen samples and having found that ILC2 proportion was unaffected by the freeze-thawing procedure (data not shown), we used freeze-thawed samples throughout the study.

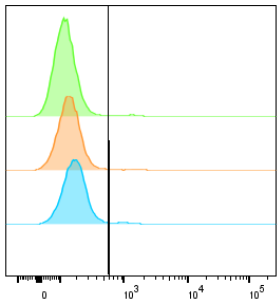
Home-made Lin- cocktail and antibody titration

Aiming to assess the proportions of all ILCs in cord blood, including ILC1s and ILC3s, we decided to use a home-made Lin- cocktail since the pre-made Lin- cocktail previously used on tonsils included the antibody anti-CD56, extracellular marker of both ILC1s and ILC3s.

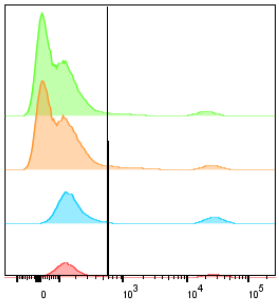
To optimize the panel, identify the correct concentration of the antibody and determine the best signal-to-noise ratio all the antibodies used in the home-made Lin- cocktail (all on FITC fluorochrome) were titrated (Fig. 16) on fresh and frozen cord blood samples starting from the dilution recommended by the producing company (Biolegend) and progressively decreased until a dilution of 1:100. The dilution showing the best separation between negative and positive populations was selected. For CD3, CD8, CD14 and CD16 the selected dilution was 1:100, for CD5, CD11c, CD19, CD20, CD34, CD123 and FCeR1a 1:50 and for CD1a 1:25.



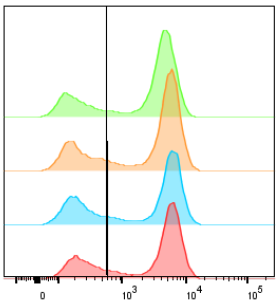
Sample Name	Subset Name	Count
CD19_1,2f,100.fcs	Lymphocytes	12619
CD19_1,2f,50.fcs	Lymphocytes	9297
CD19_1,2f,25.fcs	Lymphocytes	10824
CD19_1,2f,20.fcs	Lymphocytes	13169



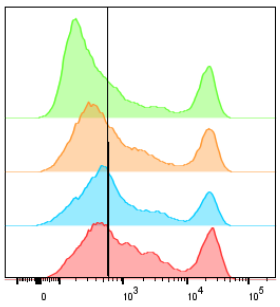
Sample Name	Subset Name	Count
cd34_1,2f,100.fcs	Lymphocytes	24597
cd34_1,2f,50.fcs	Lymphocytes	19766
cd34_1,2f,25.fcs	Lymphocytes	18053
Fresh_34_1,2f,20.fcs	Lymphocytes	20750



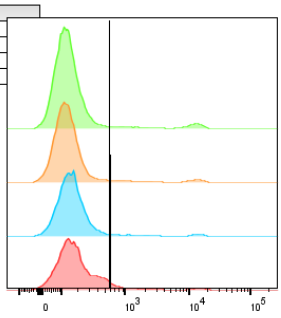
Sample Name	Subset Name	Count
CD20_1,2f,100.fcs	Ungated	103018
CD20_1,2f,50.fcs	Ungated	100795
CD20_1,2f,25.fcs	Lymphocytes	32661
CD20_1,2f,20.fcs	Lymphocytes	15866



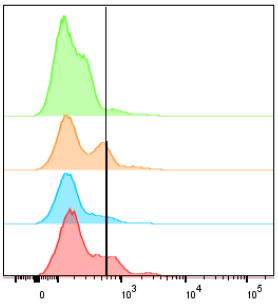
Sample Name	Subset Name	Count
cd5_1,2f,100.fcs	Lymphocytes	27200
cd5_1,2f,50.fcs	Lymphocytes	30973
cd5_1,2f,25.fcs	Lymphocytes	24882
cd5_1,2f,20.fcs	Lymphocytes	23716



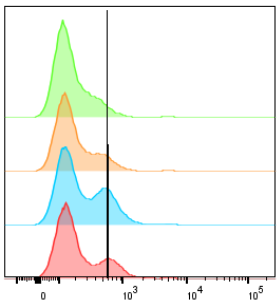
Sample Name	Subset Name	Count
cd14_1,2f,100.fcs	Lymphocytes	
cd14_1,2f,50.fcs	Lymphocytes	
cd14_1,2f,25.fcs	Lymphocytes	
cd14_1,2f,20.fcs	Lymphocytes	



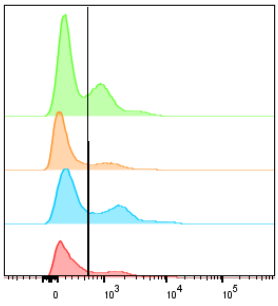
Sample Name	Subset Name	Count
cd16_1,2f,100.fcs	Lymphocytes	23408
cd16_1,2f,50.fcs	Lymphocytes	18741
cd16_1,2f,25.fcs	Lymphocytes	16105
cd16_1,2f,20.fcs	Lymphocytes	15561



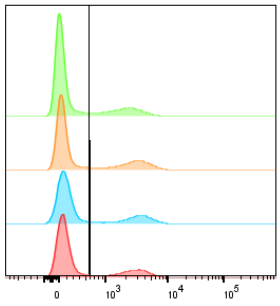
Sample Name	Subset Name	Count
FceR1a_1,2f,100.fcs	Lymphocytes	79481
FceR1a_1,2f,50.fcs	Lymphocytes	47998
FceR1a_1,2f,25.fcs	Lymphocytes	36100
FceR1a_1,2f,20.fcs	Lymphocytes	63874



Sample Name	Subset Name	Count
cd123_1,2f,100.fcs	Lymphocytes	38647
cd123_1,2f,50.fcs	Lymphocytes	32935
cd123_1,2f,25.fcs	Lymphocytes	43404
cd123_1,2f,20.fcs	Lymphocytes	34851



Sample Name	Subset Name	Count
Fresh_1a_1,2f,100.fcs	Lymphocytes	65463
Fresh_1a_1,2f,50.fcs	Lymphocytes	31503
Fresh_1a_1,2f,25.fcs	Lymphocytes	47543
Fresh_1a_1,2f,20.fcs	Lymphocytes	25565



Sample Name	Subset Name	Count
Fresh_11c_1,2f,100.fcs	Lymphocytes	53389
Fresh_11c_1,2f,50.fcs	Lymphocytes	46037
Fresh_11c_1,2f,25.fcs	Lymphocytes	41041
Fresh_11c_1,2f,20.fcs	Lymphocytes	44603

Figure 16. Titration of the antibodies for the home-made Lin- cocktail. The tested concentrations were 1:20 (red), 1:25 (blue), 1:50 (orange) and 1:100 (green).

With these dilutions we obtained a clear separation between Lin+ and Lin- populations (Fig. 17).

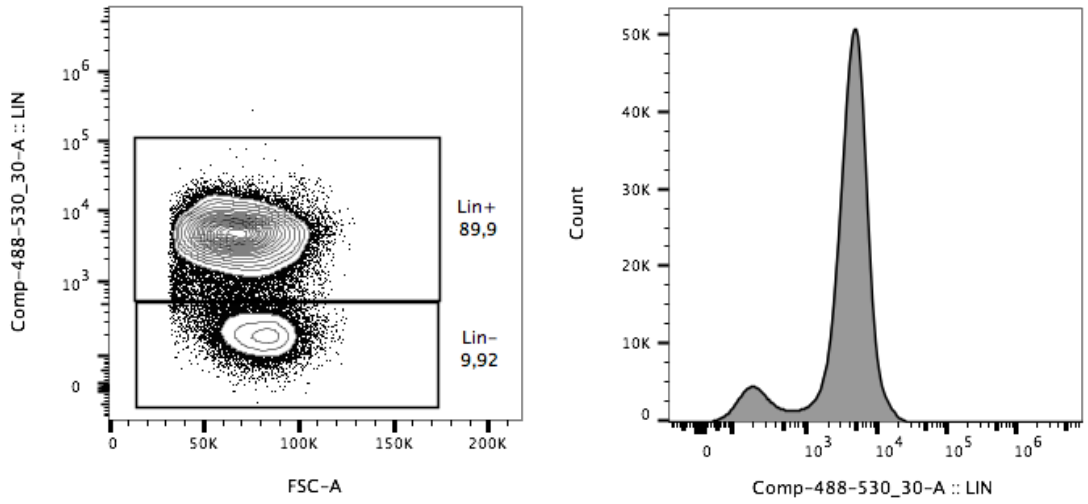


Figure 17. Flow cytometry graph of Lin+ and Lin- cell populations after staining with the home-made Lin- cocktail. Histogram representation is included.

We titrated also the other antibodies used in the panels developed to describe ILCs in cord blood. The best results were found at the following dilutions: CD45, CD127, CD56, CD8 and CD4 at 1:100; IL4, IL13, IL17, CD25, FoxP3 and CRTH2 at 1:50; CD161 and IL13 at 1:40; RorGT at 1:35; Tbet and GATA3 at 1:20.

For cord blood we developed, according to the literature [53], two new panels: one specifically describing ILCs type 1 and 3 and CD4+ lymphocytes Th1 and Th17, the other describing ILCs type 2 and CD4+ lymphocytes Th2. A parallel panel was used to assess the proportion of B cells, hematopoietic precursors, mast cells and regulatory T cells (Treg) (Fig.18).

Laser	Filter	Fluorochrome	ILC1/3	ILC2	T cells
355	**UV 450/50**	**Live/Dead**	L/D	L/D	L/D
405	Violet 450/50	BV421 (PB)	CD3	IL4	CD3
	Violet 525/50	BV505	IL17	CD3	FCeR1a
	Violet 610/20	BV605	CD45	CD45	CD45
	Violet 710/50	BV711	Tbet		
488	Blue 530/30	FITC	LIN	LIN	CD34
	Blue 695/40	PerCP5.5	CD127	CD127	
561	Yellow 585/15	PE	RORgt	IL13	FoxP3
	Yellow 610/20	PE Texas Red	CD56	CD56	CD56
	Yellow 780/60	PECy7	CD161	CD161	CD19
640	Red 670/14	APC	GATA3	GATA3	GATA3
	Red 730/45	Alexa700	IFNg	CD4	CD8
	Red 780/60	APC-Cy7	CD8	CRTH2	CD25

Figure 18. Flow cytometry panels used to describe ILC1s and ILC3s, ILC2s and T cells in cord blood.

ILC1s were described as CD45+Lin-CD161+CD56+/-CD127+Tbet+, ILC2s as CD45+Lin-CD161+CD56-CD127+CRTH2+GATA3+ and ILC3s as CD45+Lin-CD161+CD56+/-CD127+RorGT+.

To detect ILC2s in cord blood we took as example the gating strategy used by Forsberg et al. [50] (Fig. 19) but gating for CD56- cells before CD127 and CRTH2.

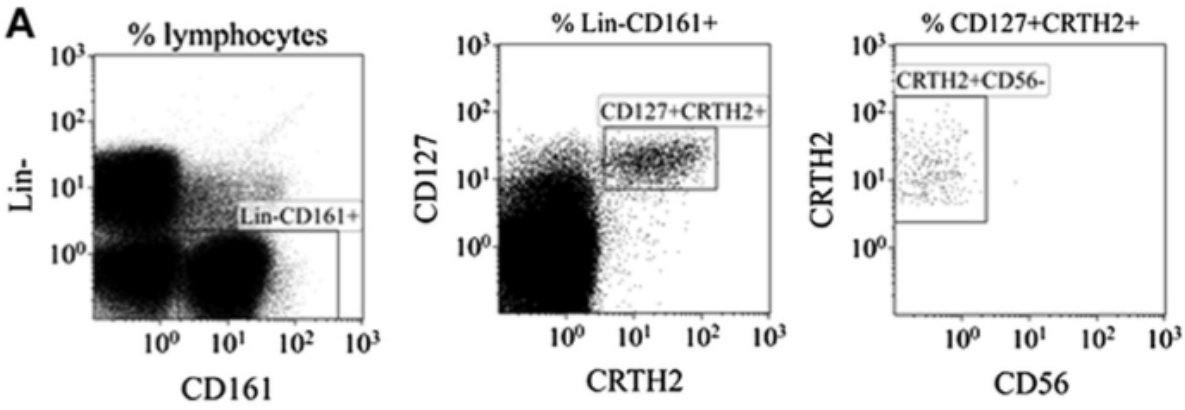
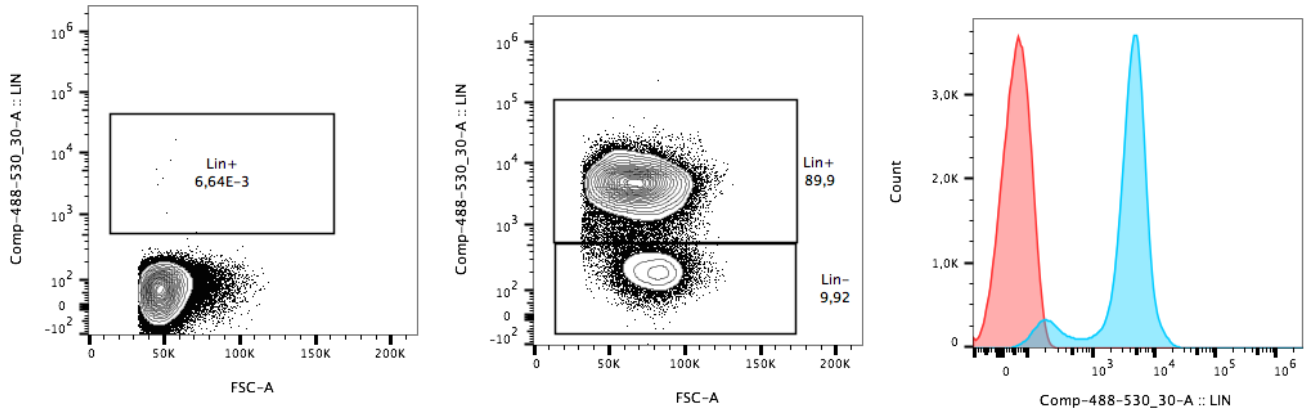


Figure 19. Gating strategy to detect ILC2s described by Forsberg [50].

A series of control samples (fluorescence minus one, FMO) were regularly prepared. These samples have one of the identifying stained omitted to allow to correctly identify positive and negative population in the test sample (Fig. 20).

A)



B)

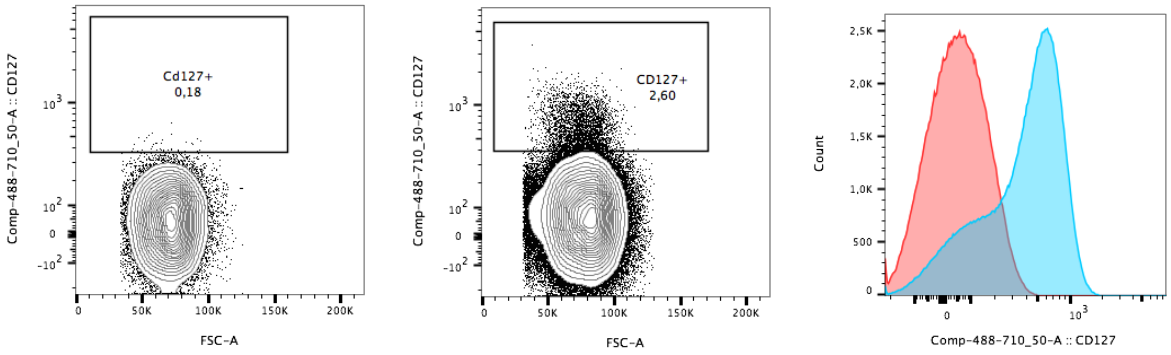


Figure 20. Examples of FMOs (A, Lin- cocktail; B, CD127) in cord blood PBMCs. On the left the FMO (i.e. the sample without the antibody of interest), in the middle the sample stained with the antibody of interest with the positive control and on the right the histogram showing the unstained and the stained samples combined.

The capacity of cord blood cells of producing cytokines was also compared with and without stimulation with PMA and Ionomycin (Fig. 21).

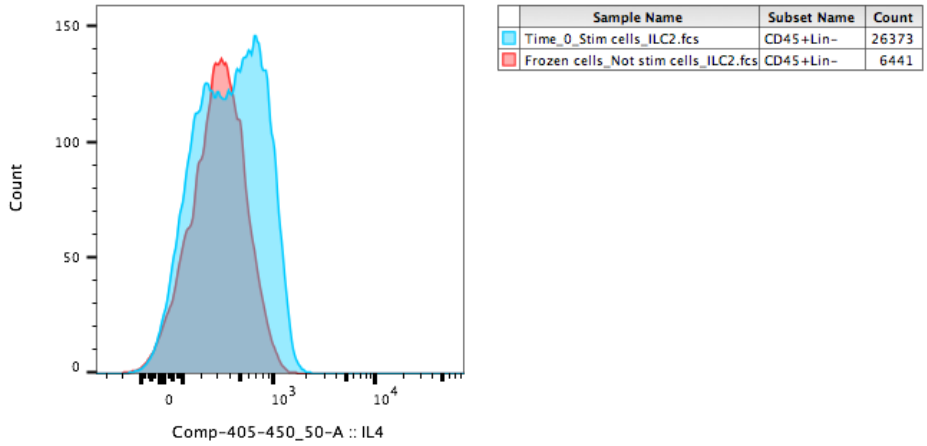


Figure 21. Histograms showing the capacity of producing IL-4 (left) with (blue peak) and without (red peak) the stimulation with PMA and Ionomycin.

On 2 cord blood samples the flow cytometry panels shown in Fig. 22 were used to assess the proportions of mast cells, basophils, eosinophils, neutrophils, monocytes and hematopoietic progenitors after LymphoPrep separation and to evaluate where these cell populations were displayed in the side scatter (SSC) – forward scatter (FSC) plot.

Laser	Filter	Fluorochrome	Panel A	Panel B
355	**UV 450/50**	**Live/Dead**	L/D	L/D
405	Violet 450/50	BV421 (PB)	CD3	CD11c
	Violet 525/50	BV505		
	Violet 610/20	BV605	CD45	CD123
	Violet 710/50	BV711	CD14	
	Violet 780/60	BV785		CD16
488	Blue 530/30	FITC	CD177	CD5
	Blue 695/40	PerCP5.5		
561	Yellow 585/15	PE	FcEr1a	
	Yellow 610/20	PE Texas Red	CD56	CD56
	670/30	PECy5		CD45
	Yellow 780/60	PECy7	CD19	
640	Red 670/14	APC	Siglec8	
	Red 730/45	Alexa700	CD1c	CD4
	Red 780/60	APC-Cy7		CD3

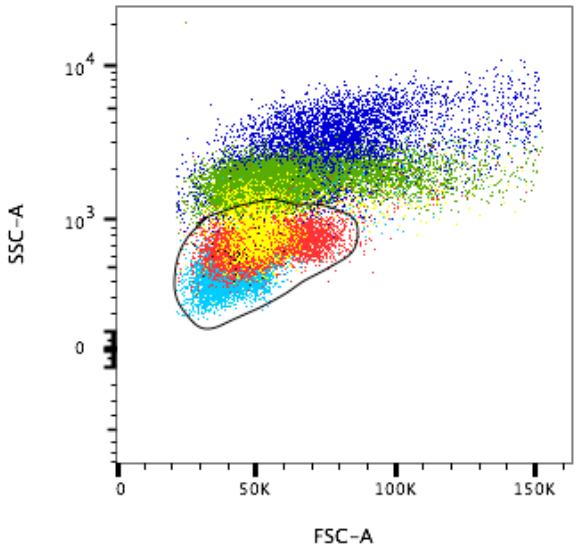
Figure 22. FACS panel used to describe the different cell population (mast cells, basophils, eosinophils, neutrophils, monocytes and hematopoietic progenitors) in cord blood after LymphoPrep separation according to the expression of extracellular markers.

Samples were run the same day of the intracellular staining on LSRFortessa III (BD). Compensation was done before any experiment. Acquisition of at least 1.500.000 events was required to obtain at least 1.000.000 lymphocytes in the FSC/SSC plot. To obtain this number we stained for each flow cytometry panel 3.5 million PBMCs. Data were analyzed by using FlowJo software v10.1.

Cord blood phenotype and gating strategy

Two samples of fresh cord blood were stained for Panel A and B after Lymphoprep separation to define where the different cell populations sat in the FSC-SSC plot and to demonstrate that the gate drawn to

detect lymphoid cells was appropriate because excluded monocytes and the potential contamination of the sample with neutrophils. With the markers used T, B and NK cells accounted for 45.3% of the sample, monocytes for 6%, neutrophils for 6.3%, mast cells and basophils for 0.56% and eosinophils for 0.01%. These findings are shown in Fig. 23.



Sample Name	Subset Name	Count
Specimen_001_	Eosinophils	65,0
Specimen_001_	Mast cells and basophils	2125
Specimen_001_	NK cells	17083
Specimen_001_	T cells	154087
Specimen_001_	Monocytes	22882
Specimen_001_	Neutrophils	24050

Figure 23. FSC-SSC plot describing the different cell populations in cord blood after LymphoPrep separation. In the drawn circle the lymphoid gate.

Starting from the selection of the lymphocytes in the FSC-SSC plot followed by the gating for alive CD45+ cells, we applied different gating strategies depending on the type of cell we wanted to detect.
According to the expression of intracellular cytokines and intranuclear transcription factors, CD4+ cells were described as lymphocytes Th1, Th17 (Fig. 24) or Th2 (Fig. 25).

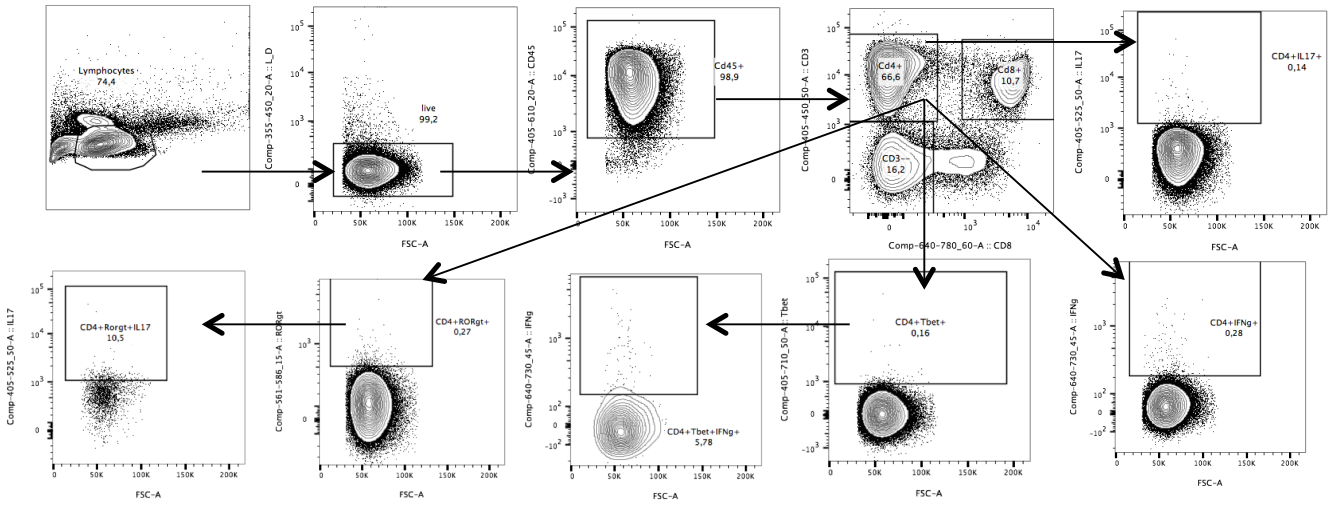


Figure 24. Gating strategy used to describe within CD4+ cells lymphocytes Th1 and Th17. Starting from the lymphoid gate where lymphoid cells can be identified for size and granularity, alive cells are selected and gated for CD45. CD4+ cells are then selected and gated for: those able to produce IL-17 and IFNg; those positive for the trascription factors Tbet+ and RorGT+.

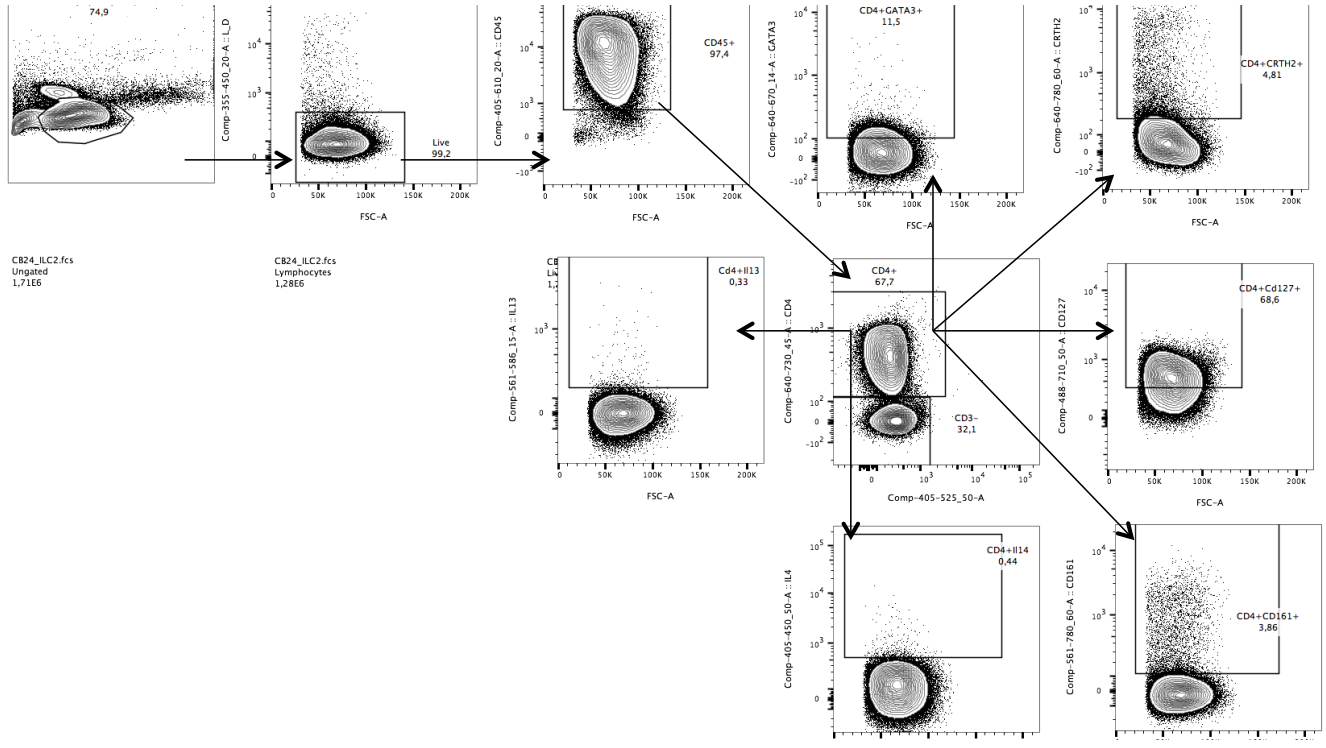


Figure 25. Gating strategy used to describe within CD4+ cells lymphocytes Th2. Starting from the lymphoid gate where lymphoid cells can be identified for size and granularity, alive cells are selected and

gated for CD45. CD4+ cells are then selected and gated for: those able to produce IL-4 and IL-13; those positive for the trascription factor GATA3+; those positive for CD127+, CRTH2+ or CD161+.

Panel ILC1/3 allowed recognizing ILC1s (CD45+Lin-CD161+CD56+/-CD127+Tbet+, capable of producing IFNg) and ILC3s (CD45+Lin-CD161+CD56+/-CD127+RorGT+, capable of producing IL17). Both cell types are thought to have two subtypes depending on the expression of CD56+. The gating strategy is demonstrated in Fig. 26.

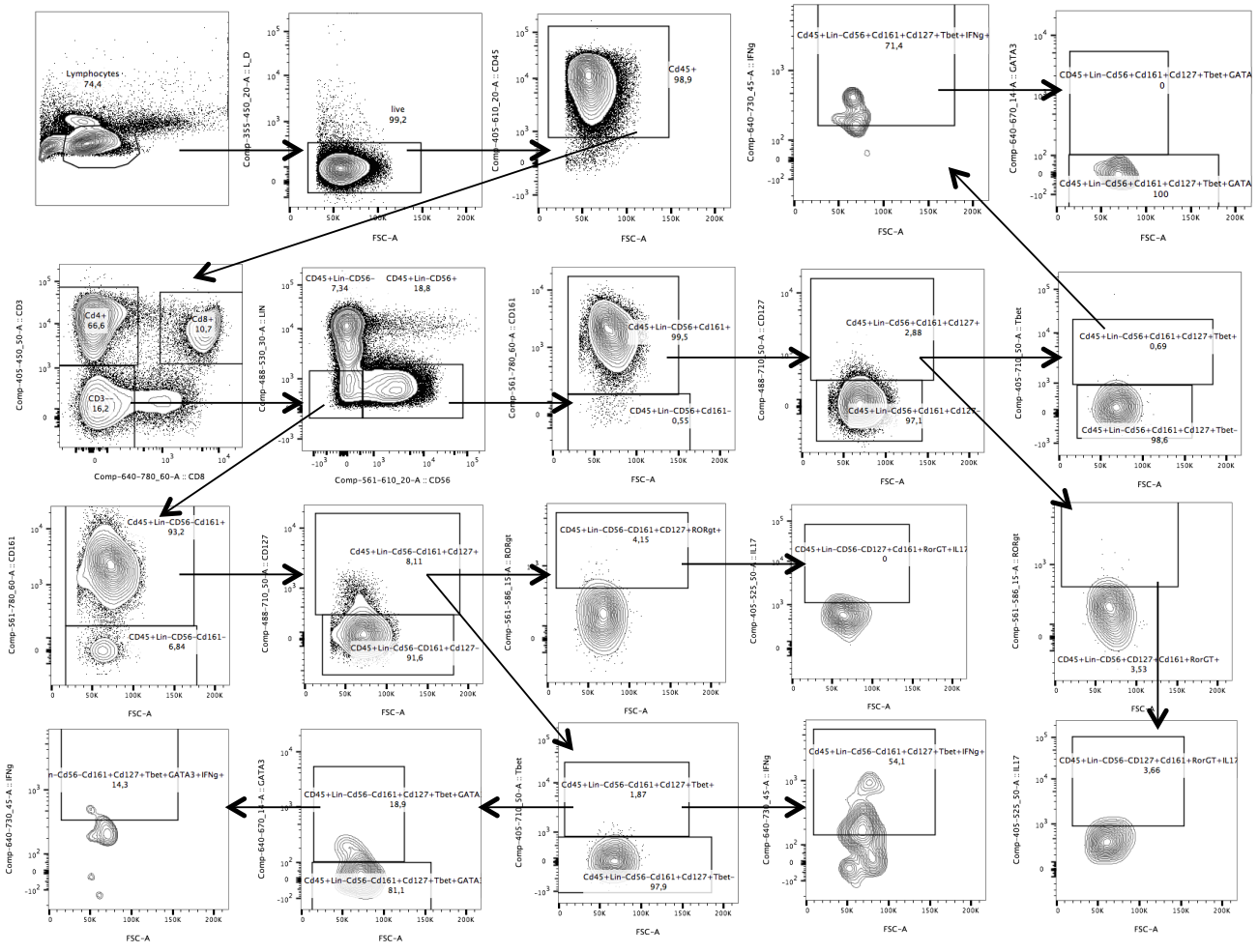


Figure 26. Gating strategy used to describe ILC1s (CD45+Lin-CD56+/-CD161+CD127+Tbet+) and ILC3s (CD45+Lin-CD56+/-CD161+CD127+RORgt+). Starting from the lymphoid gate where lymphoid cells can be identified for size and granularity, alive cells are selected and gated for CD45. CD3+ cells are then excluded and the remaining gated for the Lin- cocktail and CD56. From this point both Lin-CD56+ cells

and Lin-CD56- cells are gated for CD161, then for CD127 and finally for the transcription factors Tbet or RorGT and for the respective interleukins IFNg and IL-17.

ILC2s (CD45+Lin-CD161+CD56-CD127+CRTH2+GATA3+, capable of producing IL4 and IL13) have been described with Panel ILC2. After the initial gates selecting CD45+ alive lymphoid cells, the used gating strategy is shown in Fig. 27.

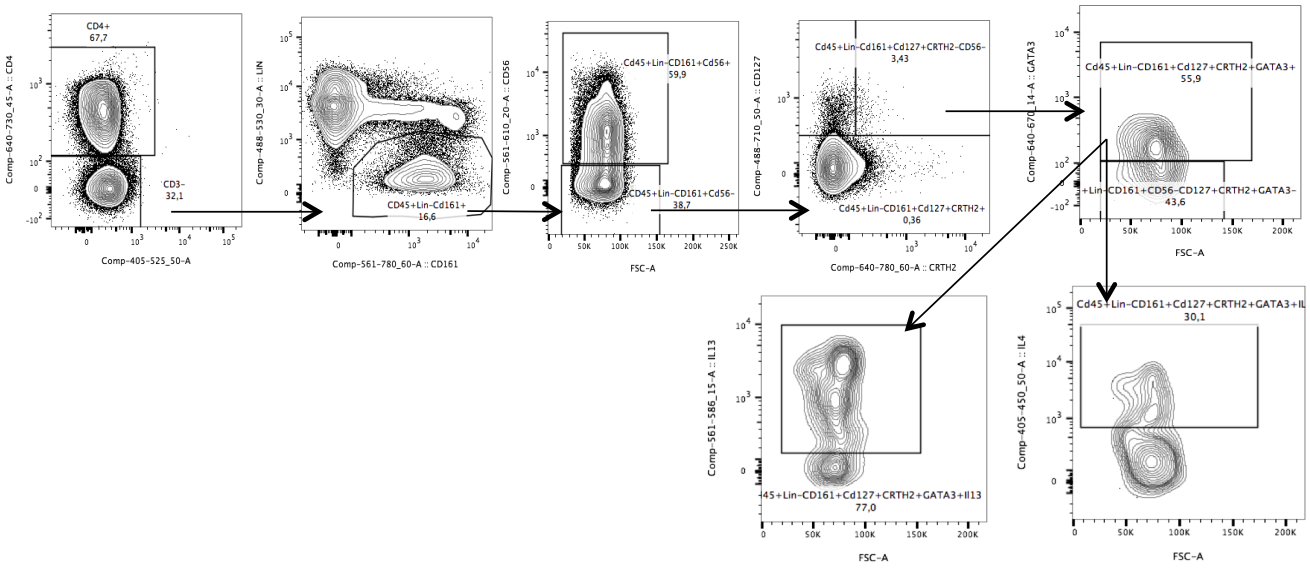


Figure 27. Gating strategy used to describe ILC2s (CD45+Lin-CD161+ CD56-CD127+CRTH2+GATA3). Starting from the lymphoid gate where lymphoid cells can be identified for size and granularity, alive cells are selected and gated for CD45 (graphs not shown). CD3+ cells are excluded and the remaining cells gated for the Lin- cocktail and CD161. Lin-CD161+ cells negative for CD56 are gated for CD127 and CRTH2 and the resulting fraction gated for the transcription factor GATA3 and then for the interleukins IL-4 and IL-13.

Cord blood samples and demographic factors of the population

Twenty samples of cord blood from at term elective caesarian section were collected (10 males). Table 1 summarizes cord blood characteristics and baby and mother features.

	Mean (SD) or median (range)
Blood	11.1 ml (3.5)
PBMCs/ml	6.6 million (3.2-16)
M/F	10/10
Birth weight	3277.1 g (302.5)
Gestational age	38.4 weeks (0.6)
Maternal age	34.3 years (5.0)
Maternal blood eosinophil count	0.05 x 10^9/L (0.08)
Atopic mothers	6/20
Smoking habit	1/20

Table 1. Cord blood characteristics and baby and mother features.

Cord blood CD45+ cells

T cell and ILC proportions were described as percentage (%) of alive CD45+ lymphoid cells.

In cord blood the most abundant CD45+ cell populations found within the lymphoid gate were T cells (71% SD:9.3%), B cells (14.8% SD:5.9%) and NK cells (4.6% SD:3.5%). NKT cells (CD3+CD56+), hematopoietic progenitors (CD45+34+ and CD45-CD34+) and CD45+FcEr1a+ cells (mast cells, eosinophils and basophils) were also estimated (Fig. 28). The latter cell population (CD45+FcEr1a+) was measured because the back gating strategy displayed in Figure 22 showed that these cells could sit within the lymphoid gate and we wanted to quantify their contamination (0.2% SD:0.17%). Anti-human FcEr1a antibody was included in the home-made Lin- cocktail.

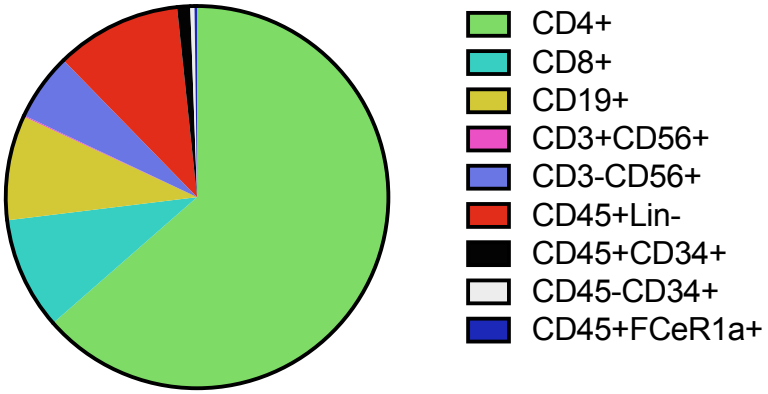


Figure 28. Cord blood cellularity. T cells (CD4+ 54.5% SD:8.2% and CD8+ 17% SD:5%), B cells (CD19+) 14.8% SD:5.9, NK T cells (CD3+CD56+) 0.1% SD:0.08%, NK cells (CD3-CD56+) 4.6% SD:3.5%, Lin- cells (CD45+Lin-) 8.3% SD:4.3%, cell precursors (CD45+CD34+ 2% SD:1.4 and CD45-CD34+ 1.1% SD:0.7), mast cells (CD45+FCeR1a+) 0.2% SD:01%.

Compared to CD4+ cells, CD45+Lin- cells were significantly fewer (Fig. 29).

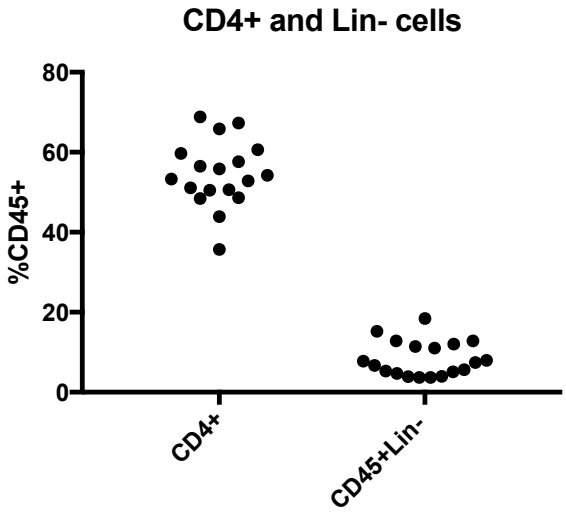


Figure 29. CD4+ cells and CD45+Lin- cells in cord blood. Values expressed as % of alive CD45+ lymphoid cells.

ILC1s (CD45+Lin-CD56+/-CD161+CD127+Tbet+) were the most numerous sub-type of ILCs present in cord blood followed by ILC2s (CD45+Lin-CD161+CD56-CD127+CRTH2+GATA3+) (ILC1s 0.033% SD:0.04%; ILC2s 0.015% SD:0.012%) (Fig. 30).

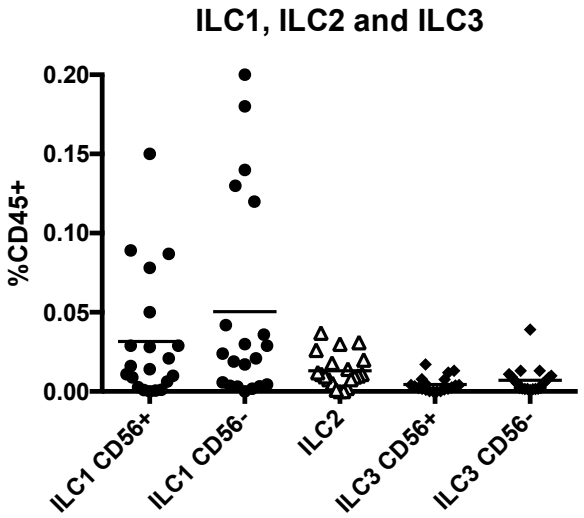


Figure 30. Percentages of ILCs in cord blood mononuclear cells. ILC1s, CD45+Lin-CD161+CD127+Tbet+ (subgroups CD56+ and CD56-); ILC2s, CD45+Lin-CD161+CD56-CD127+CRTH2+GATA3+; ILC3s, CD45+Lin-CD161+CD127+RorGT+ (subgroups CD56+ and CD56-). Values expressed as % of alive CD45+ lymphoid cells.

All ILCs (type 1, 2 and 3) were capable of producing the respective interleukins. Depending on ILC type, ILC1s can be positive for IFNg, ILC2s for IL4 and/or IL13, and ILC3s for IL17. Figure 31 shows the proportions of all ILCs with those able to produce interleukins (all expressed as % of alive CD45+ lymphoid cells).

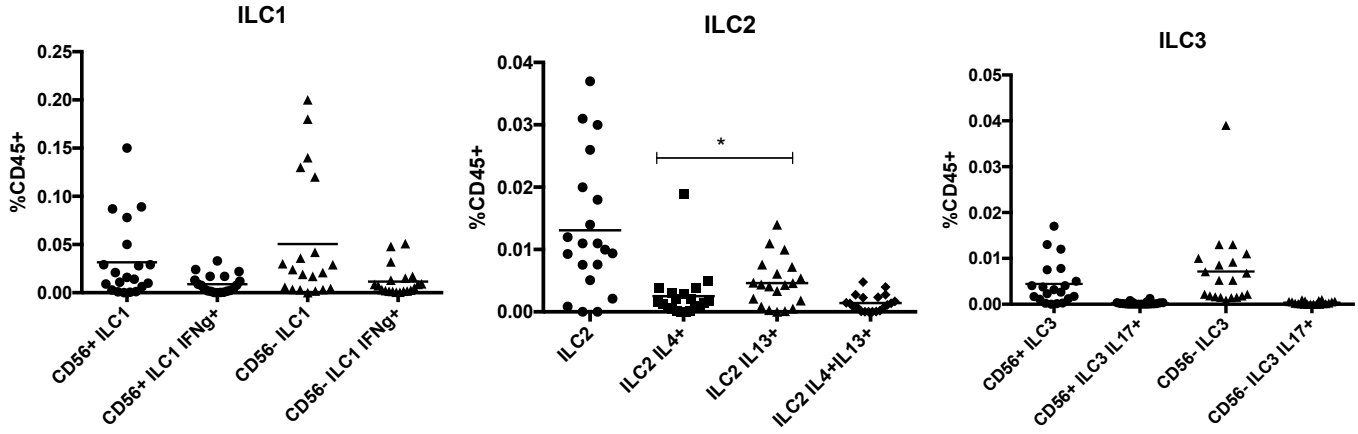


Figure 31. Proportions of ILCs type 1, 2 and 3 and proportions of those positive for the associated interleukins. A) Proportions of ILCs type 1 (CD45+Lin-CD161+CD127+Tbet+) divided in the 2 subgroups (CD56+ and CD56-). For each group it is also plotted the proportion of cells positive for IFNg; B) Proportions of ILCs type 2 (CD45+Lin-CD161+CD56-CD127+CRTH2+GATA3+) and proportions of

ILC2s positive for IL-4, IL-13 or both; C) Proportions of ILCs type 1 (CD45+Lin-CD161+CD127+RorGT+) divided in the 2 subgroups (CD56+ and CD56-). For each group it is also plotted the proportion of cells positive for IL-17. Values expressed as % of alive CD45+ lymphoid cells. Data were analysed with t-test. * $p < 0.05$.

Considering the rude number of each ILC type, we found that: about one third of both subsets of ILC1s were capable of producing IFNg (CD56+ILC1: 32.9% SD:21.7%; CD56-ILC1: 31.7% SD:23.5%), ILC2s showed a greater capacity of producing IL-13 rather than IL-4 (36.9% SD:23.2% vs 14.1% SD:11.7%, $p < 0.05$), and ILC3s had different ability of secreting IL-17 depending on subset (CD56+ILC3: 11.9% SD:17.4%; CD56-ILC3 6.1% SD:5.9%) (Fig. 32).

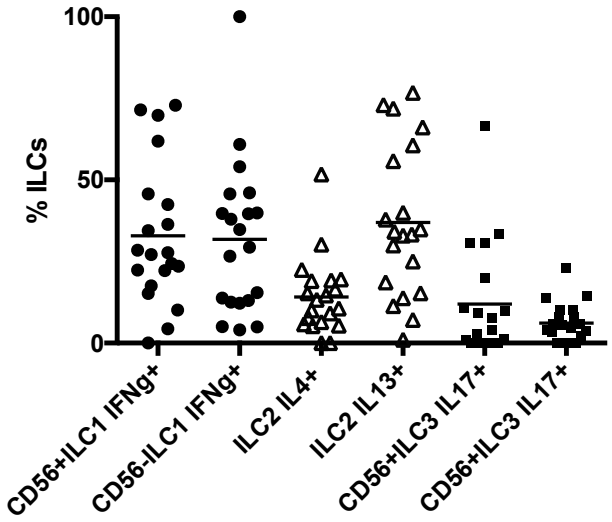
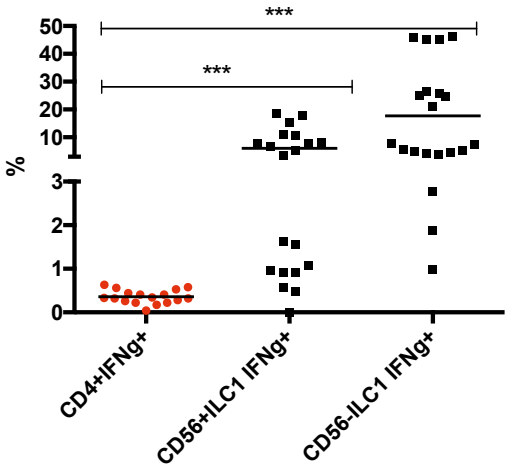


Figure 32. Percentages of different ILC types capable of producing the associated interleukins.
The percentage has been calculated referring to the total rude number of each type of ILC (i.e. n° of ILC2s positive for IL4 within the total number of ILC2s).

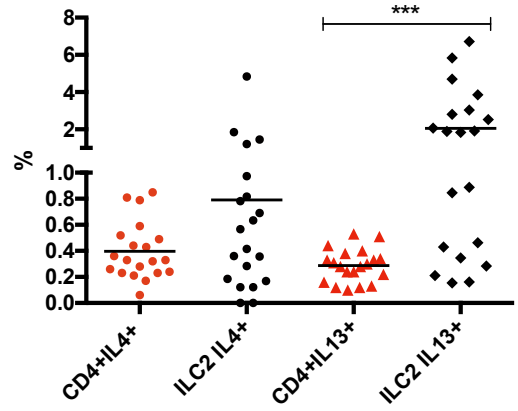
In order to compare the capacity of ILCs (CD45+Lin-CD56+/-CD161+CD127+) and CD4+ cells to produce interleukins, the number of ILCs type 1, 2 and 3 positive for the respective intra-cellular cytokines were analysed as a percentage of CD45+Lin-CD56-CD161+CD127+ for ILC2s and for the CD56- subgroups of ILC1s and ILC3s and as a percentage of CD45+Lin-CD56+CD161+CD127+ for the CD56+ subgroups of ILC1s and ILC3s.
Compared to CD4+ cells, a greater percentage of ILC1s (both CD56+ and CD56- subgroups) and ILC2s were capable of producing IFNg (6.04% SD:6.0 vs 0.35% 0.15) and IL-13 (1.96% SD:1.94 vs 0.27%

SD:0.11) (Fig 33A and B) respectively while ILC3s, both CD56+ and CD56- subgroups, had less capacity of producing IL-17 than CD4+ cells (Fig. 33C).

A)



B)



C)

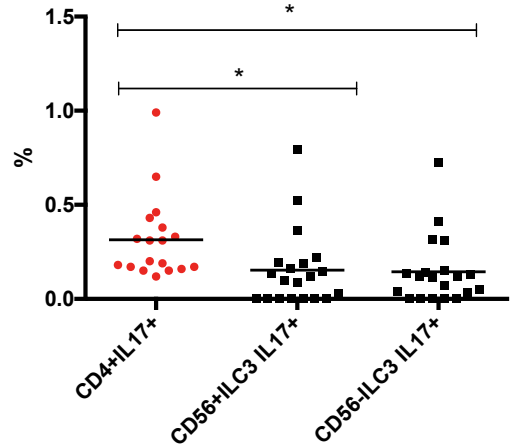


Figure 33. Percentages of different cells capable of producing the associated interleukins: A) IFNg in CD4+ cells vs ILC1s (CD56+ and CD56- subgroups); B) IL-4 and IL-13 in CD4+ cells vs ILC2s; C) IL-17 in CD4+ cells vs ILC3s (CD56+ and CD56- subgroups).

The percentage has been calculated referring to the total rude number of each type of cell.

% stands for % of CD4+ cells when referring to CD4+ cells positive for IFNg, IL-4, IL-13 or Il-17 whereas stands for % of CD45+Lin-CD56+/-CD161+CD127+ when referring to ILC1s, ILC2s and ILC3s positive for IFNg, IL-4, IL-13 and IL-17. Data were analysed with t-test. * $p < 0.05$; *** $p < 0.005$.

CD4+ cells were significantly higher in cord blood than ILC2s (54.19% SD:8.96% vs 0.015% SD:0.012%) (Fig 34).

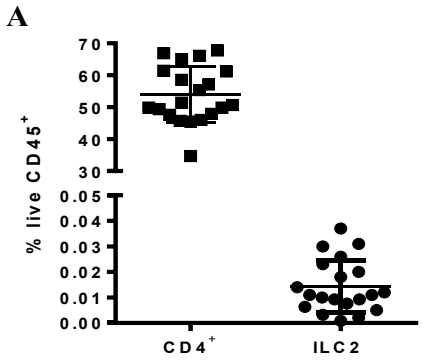


Figure 34. Percentage of CD4+ cells and ILC2s in cord blood. Values expressed as % of alive CD45+ lymphoid cells.

ILC2s interleukins and their proportion in newborn boys and girls

ILC2s were more capable of producing IL-13 than IL-4 (33.7% SD:21.2% vs 12.1% SD:10.9%; $p < 0.05$) as demonstrated by Fig. 35 where proportions of all ILC2s, ILC2s positive for IL-4 or IL-13 and ILC2s positives for both IL-4 and IL-13 are shown.

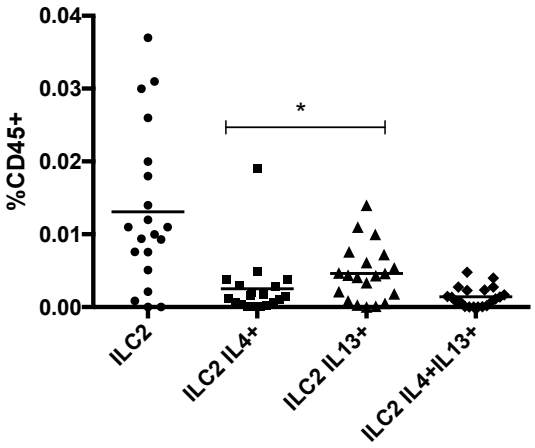


Figure 35. Proportions of ILC2s (all ILC2s, ILC2s positive for IL-4 or IL-13, ILC2s positive for both IL-4 and IL-13) expressed as percentage of alive CD45+ lymphoid cells.

Compared to girls, newborn boys had a greater number of ILC2s (CD45+Lin-CD161+CD56-CD127+CRTH2+GATA3+) (0.008% SD:0% vs 0.019% SD:0.01%; $p < 0.05$) regardless of maternal atopic status and maternal peripheral blood eosinophil count (Fig. 36).

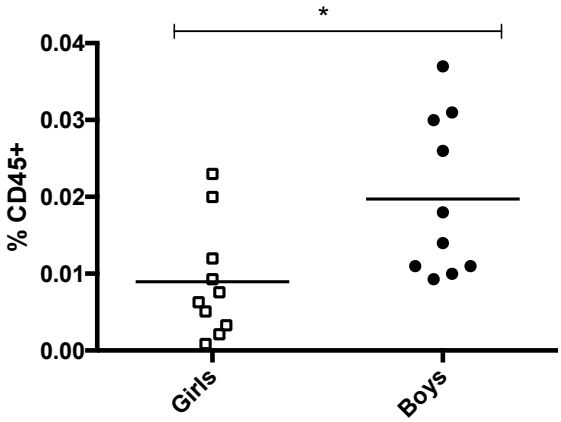
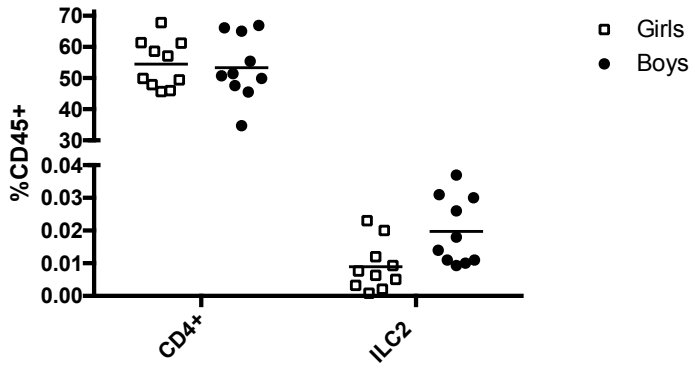


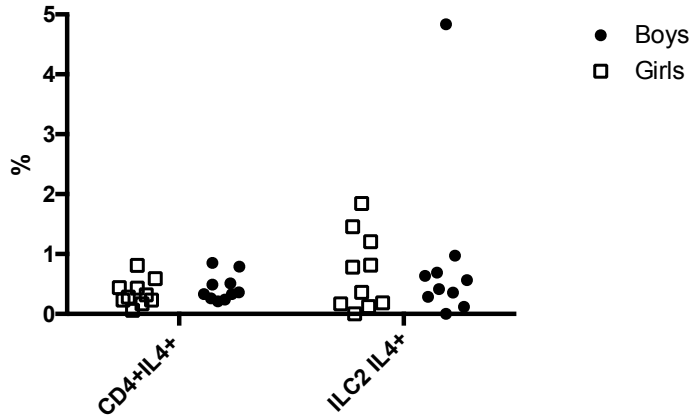
Figure 36. Proportions of ILC2s expressed as percentage of alive CD45+ lymphoid cells in girls and boys. * $p < 0.05$.

No difference was demonstrated between girls and boys in the number of CD4+ cells or in the capacity of producing IL-4 and IL-13 (Fig. 37).

A)



B)



C)

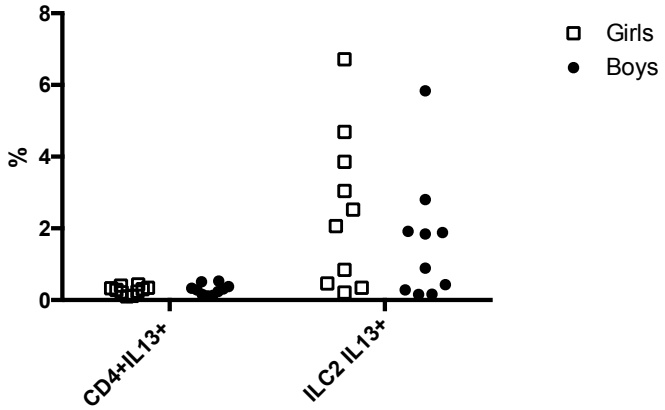


Figure 37. Comparison between girls and boys. A) Proportions of CD4+ cells and ILC2s expressed as % of alive CD45+ lymphoid cells; B and C) Proportions of CD4+ cells and ILC2s positive for IL4 (B) and IL13 (C).

In B and C graphs values are expressed as % of CD4+ cells (A) and % of CD45+Lin-CD161+CD56-CD127+ cells respectively (B and C).

No correlation was found between the number of ILC2s and birth weight or gestational age.

Sorting of ILC2 and CD4+ cells and cell culture

ILC2 sorting was performed on 3 samples of cord blood of which 2 previously frozen (n 1 and n 2) and 1 fresh (n 3), and on 1 sample of peripheral blood, the latter both fresh and frozen.

The fresh samples were kept in a humidified incubator overnight and sorted the day after. ILC2s were sorted as CD45+Lin-CD161+CD56- (see the flow cytometry panel for sorting). A parallel sorting was performed on CD4+ cells.

The median number of cord blood PBMCs (n 4 samples) entering the sorting was 10 x 10^6 (7-40) of which 45% SD:2.8% was recognised as lymphocytes on FSC-SSC plot. Possible ILC2s (CD45+Lin-CD161+CD56-) formed 0.58% SD:0.43% of alive CD45+ lymphoid cells with a median absolute number of sorted cells of 16.700 (8.423-23.123) (output number). No difference was found between the frozen and the fresh sample in the percentage of ILC2s obtained with the sorting.

The sorted cells were cultured in supplemented medium (RPMI 1640 medium containing L-glutamine 300 mg/L, 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml), 10% fetal calf serum, 1% Hepes 0.5 M, 1% essential amino acids, 1% sodium pyruvate and 1% glutamine) at a concentration of about 10.000 cells/well in a 96 well plate. Cells were stimulated with IL-2 (100 UI/ml), IL-7 (50 UI/ml) and IL-33 (50 UI/ml) (IL cocktail) ± Budesonide (10^{-8} M) and harvested at day 1, 3, 5 or 7 depending on the experiments.

Viability of ILC2 and CD4+ cultures in the different samples at different time points is shown in Fig. 38. Leaving the cells in culture seemed to promote their viability but since the datum is expressed as a percentage of cells in the FSC-SSC plot we argue that it is biased by a progressive reduction in the total number of cells (cell aggregates due to sorting may be changed to debris and therefore not included in the lymphoid gate in the FSC-SSC plot).

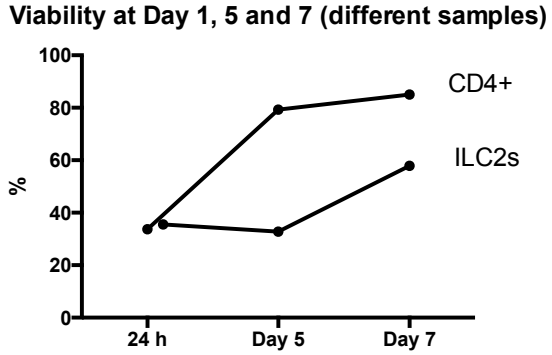


Figure 38. Percentage (%) of viable cells at different time points for CD4+ and ILCs considering 3 different samples indicated by the 3 points.

In the ILC2 culture ILC2 capacity of producing IL-4 and IL-13 reached the peak at 24 hours and then decreased on day 5 and 7. CD4+ cell culture showed similar results (Fig. 39).

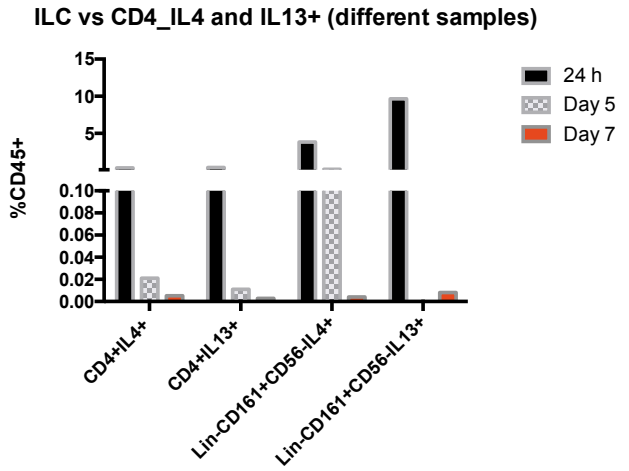


Figure 39. Proportions of sorted CD4+ cells and sorted ILC2s capable of producing IL-4 and IL-13 in the respective cultures at different time points (24 h, Day 5 and Day 7). Values expressed as % of alive CD45+ lymphoid cells.

During the ILC2 culture the fraction of Lin+ cells progressively increased while the fraction of the Lin- decreased as shown in Fig. 40.

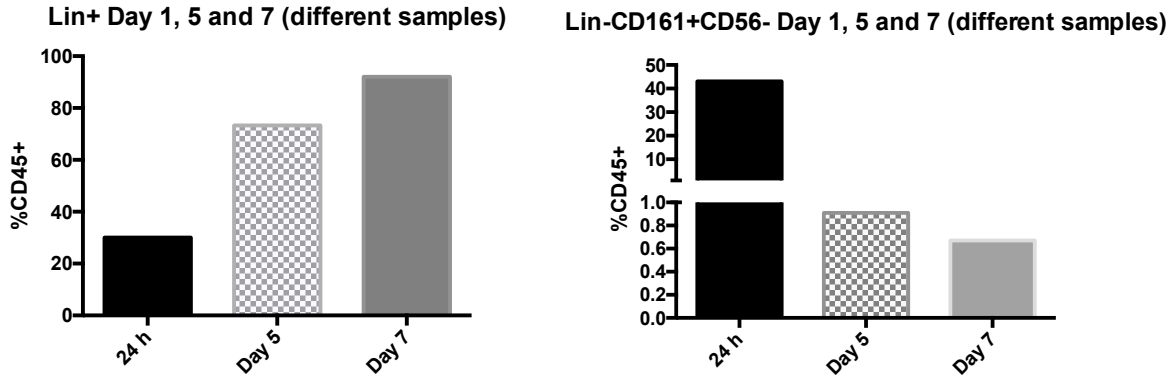


Figure 40. Lin+ (left) and Lin-CD56-CD161+ (right) fractions in the culture of sorted ILCs at 3 time points (24 h, Day 5 and Day 7). Each column represents a different sample. Values expressed as % of alive CD45+ lymphoid cells.

ILC2s and Budesonide

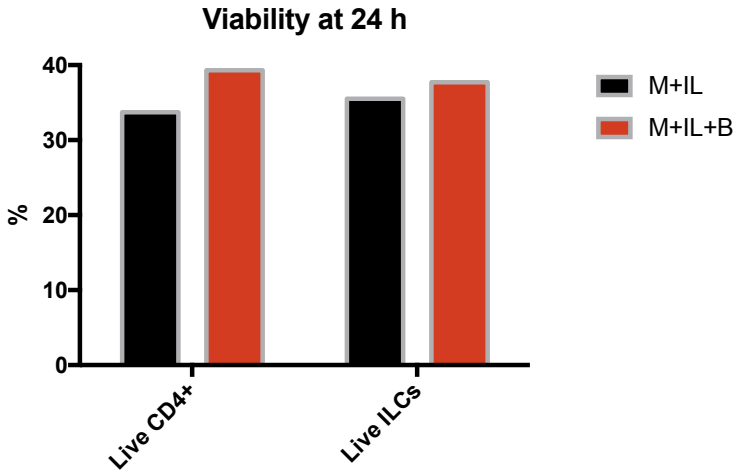
We hypothesized that ILC2s might be steroid resistant. To assess the effect of Budesonide on ILC2s, in 2 experiments the sorting of fresh cord blood PBMCs gave more than 20.000 ILC2s and therefore we could plate 10.000 ILC2s/well in 2 wells to assess the effect of Budesonide on these cells. The chosen concentration of Budesonide (10^{-8} M) was based on the literature (51) and on previous experiences in the

laboratory. A parallel culture of sorted CD4+ cells (10.000 cells/well) was done in the same conditions. Cells were sorted and left in supplemented medium in a humidified incubator overnight. The day after 75% of the medium was discarded (after having centrifuged the plate for 1 minute at 300 RPM) and the wells refilled as follows: 1 well was filled with supplemented medium and IL-2 (100 UI/ml), IL-7 (50 UI/ml) and IL-33 (50 UI/ml) and 1 well with supplemented medium, interleukins and Budesonide 10^{-8} M.

Cells were harvested and analysed by flow cytometry 24 hours later.

Cell viability did not differ between ILC2s and CD4+ cells or in the 2 culture conditions (Fig. 41).

A)



B)

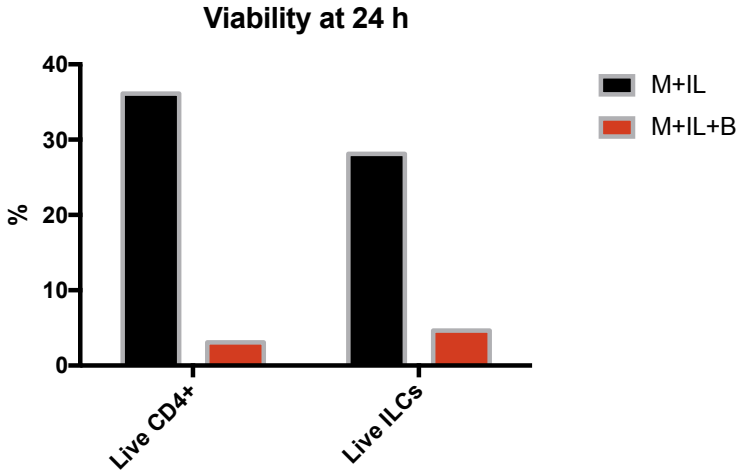


Figure 41. Cell viability (expressed as %) at 24 hours in the culture of sorted CD4+ cells and sorted ILC2s in the two experiments performed (A and B).

M+IL: supplemented complete medium + interleukins (IL-2, IL-7, IL-33).

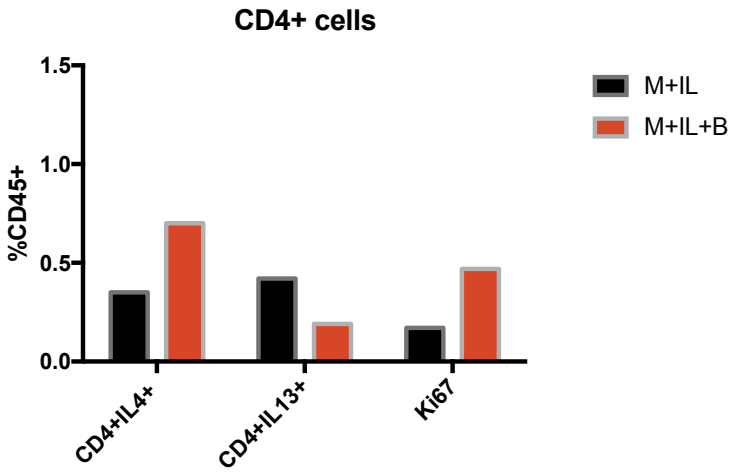
M+IL+B: medium + interleukins (IL-2, IL-7, IL-33) + Budesonide 10^{-8}M.

Supplemented complete medium: RPMI 1640 medium containing L-glutamine 300 mg/L, 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml) and 10% fetal calf serum (FCS) supplemented with 1% Hepes 0.5 M, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate.

Cytokines were added to the culture with the following concentrations: IL-2, 100 UI/ml; IL-7, 50 UI/ml; IL-33, 50 UI/ml.

In the two experiments performed, CD4+ cells seemed to proliferate more (increase of Ki67) and to be able to produce greater quantity of IL-4 in the Budesonide condition (Fig. 42).

A)



B)

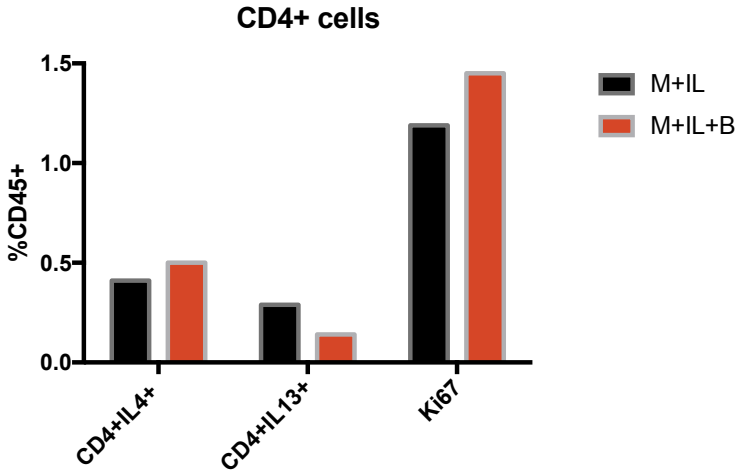


Figure 42. Capacity of producing IL-4 and IL-13 in the culture of sorted CD4+ cells in the two experiments performed (A and B). Ki67 represents an index of cell proliferation.

M+IL: supplemented complete medium + interleukins (IL-2, IL-7, IL-33).

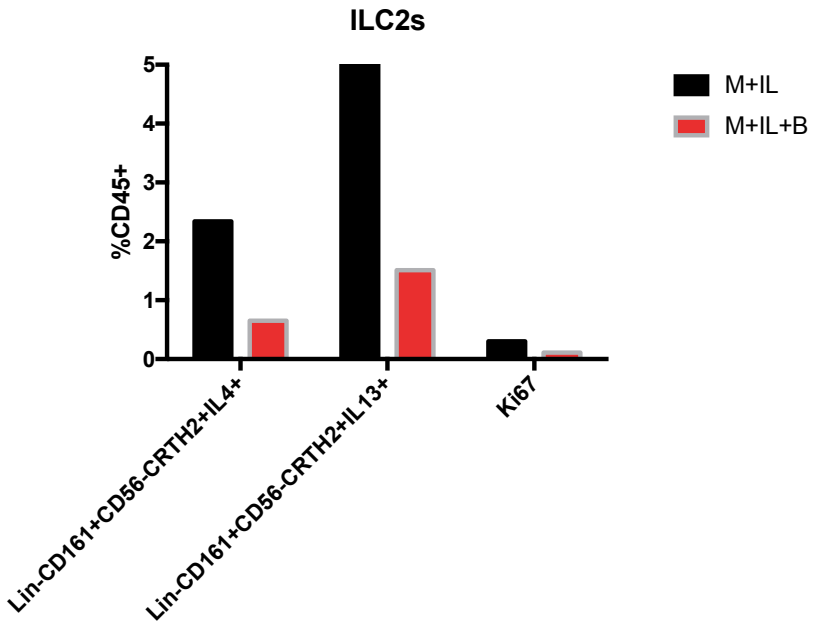
M+IL+B: medium + interleukins (IL-2, IL-7, IL-33) + Budesonide 10^{-8}M.

Supplemented complete medium: RPMI 1640 medium containing L-glutamine 300 mg/L, 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml) and 10% fetal calf serum (FCS) supplemented with 1% Hepes 0.5 M, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate.

Cytokines were added to the culture with the following concentrations: IL-2, 100 UI/ml; IL-7, 50 UI/ml; IL-33, 50 UI/ml.

On the contrary, ILC2s in presence of Budesonide reduced their proliferation and the capacity of producing interleukins (Fig. 43).

A)



B)

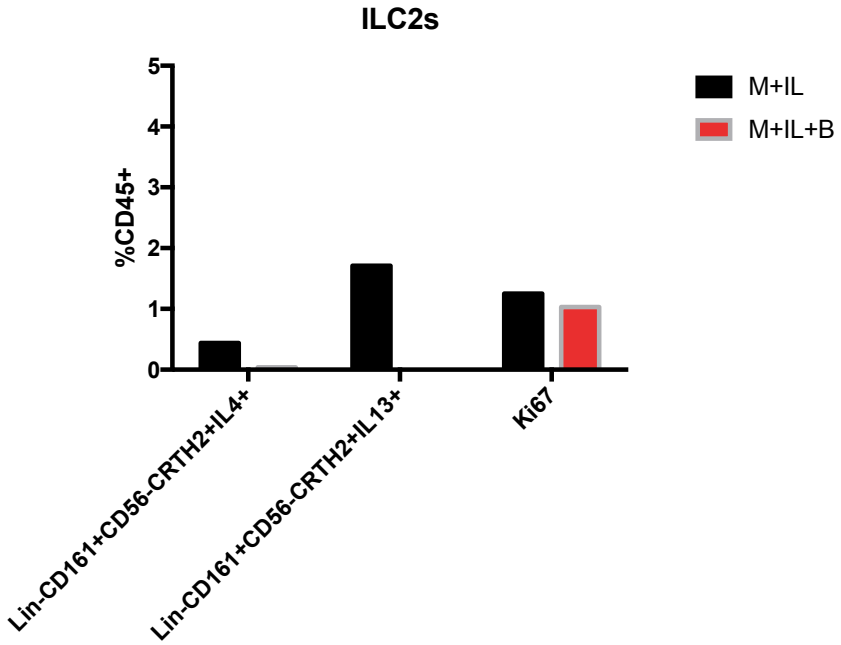


Figure 43. Capacity of producing IL-4 and IL-13 in the culture of sorted ILC2s in the two experiments performed (A and B). Ki67 represents an index of cell proliferation

M+IL: supplemented complete medium + interleukins (IL-2, IL-7, IL-33).

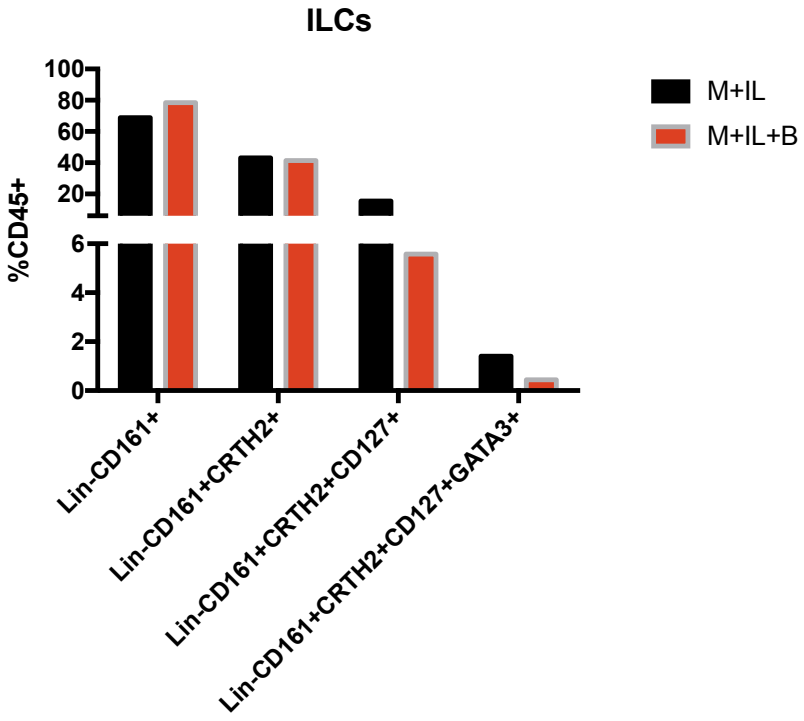
M+IL+B: medium + interleukins (IL-2, IL-7, IL-33) + Budesonide 10^{-8}M.

Supplemented complete medium: RPMI 1640 medium containing L-glutamine 300 mg/L, 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml) and 10% fetal calf serum (FCS) supplemented with 1% Hepes 0.5 M, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate.

Cytokines were added to the culture with the following concentrations: IL-2, 100 UI/ml; IL-7, 50 UI/ml; IL-33, 50 UI/ml.

The expression of ILC2 markers in the 2 conditions (supplemented complete medium added with interleukins with and without Budesonide) is shown in Fig. 44.

A)



B)

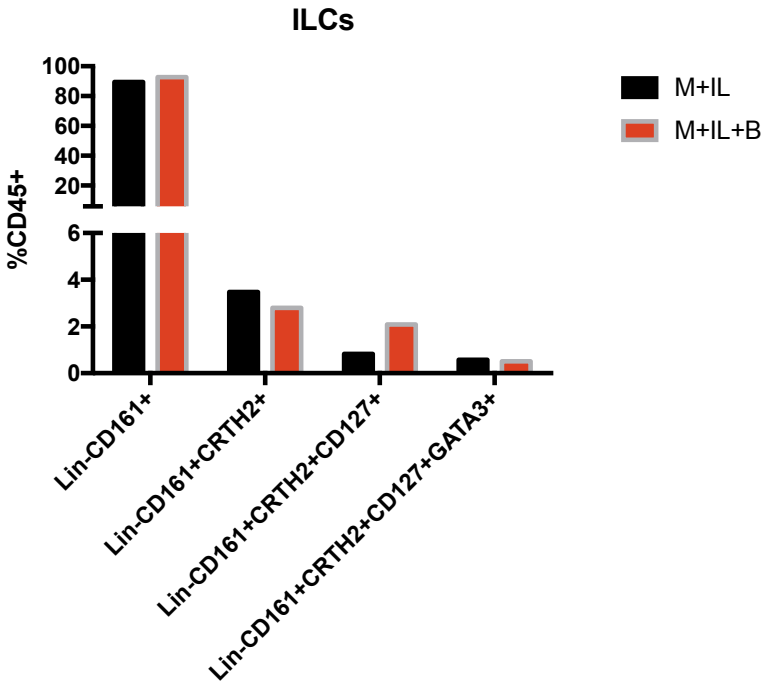


Figure 44. Expression of different ILC2 markers with and without Budesonide in the two experiments performed (A and B).

M+IL: supplemented complete medium + interleukins (IL-2, IL-7, IL-33).

M+IL+B: medium + interleukins (IL-2, IL-7, IL-33) + Budesonide 10^{-8}M.

Supplemented complete medium: RPMI 1640 medium containing L-glutamine 300 mg/L, 1% Penicillin (100 U/ml), 1% Streptokinase (100 ug/ml) and 10% fetal calf serum (FCS) supplemented with 1% Hepes 0.5 M, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate.

Cytokines were added to the culture with the following concentrations: IL-2, 100 UI/ml; IL-7, 50 UI/ml; IL-33, 50 UI/ml.

Considering the fraction of Lin+ cells in the sorted ILC2s, we found that the contamination from CD4+ cells was about 1% of alive CD45+ lymphoid cells (M+IL 1%; M+IL+B 0.98%) and that a certain number of cells have possibly become positive for FcEr1a, marker for basophils and mast cells (M+IL: 1.78%; M+IL+B: 0.93%).

Peripheral blood sorting

The median number of peripheral blood PBMCs entering the sorting was 20 x 10^6 SD:7 x 10^6 of which 19.4% SD:6.1% were lymphocytes. ILCs (CD45+Lin-CD161+CD56-) formed 0.21% SD:0.02% of alive lymphocytes with a mean rude number of sorted cells of 6.152 SD:3385.6. ILCs and CD4+ cells were

cultured in a 96 well plate (up to 10.000 cells /well); ILC2s were cultured for 3 days and CD4+ cells for 5 days with harvesting at day 3 and 5.

Whereas no difference was found between the frozen and the fresh sample in the percentage of sorted ILCs, cell viability at day 3 was lower in the frozen sample (25% vs 35.1%). Similar findings were reported for CD4+ cells (cell viability at day 5: fresh sample 71%, frozen sample 61%).

Figure 45 shows the capacity of sorted CD4+ cells of producing IL-4 and IL-13 at day 3 and 5.

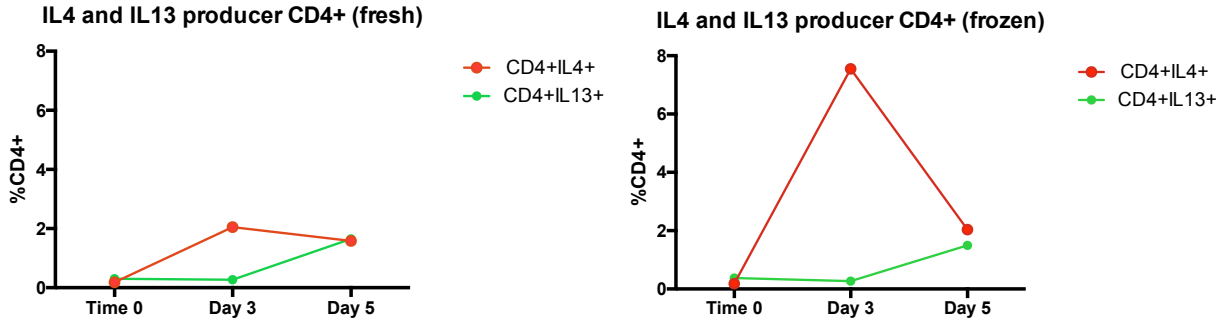


Figure 45. Capacity of sorted CD4+ cells of producing IL-4 and IL-13 at different time points. Time 0 represents the sample before sorting. Cell numbers are expressed as % of CD4+ cells.

Flow cytometry analysis of sorted ILC2s at day 3 showed greater proportions of ILC2s in the fresh sample with a preserved capacity of producing interleukins (Fig. 46).

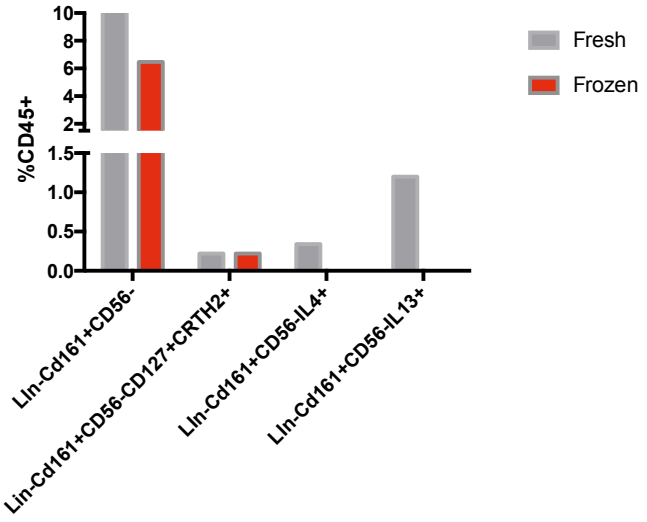


Figure 46. Proportion of ILC2s (subdivided in different groups depending on markers expression) at day 3 of sorted ILC2 culture in a fresh and a frozen sample.

Cytospins of sorted ILC2s and CD4+ cells were also performed (Fig. 47).

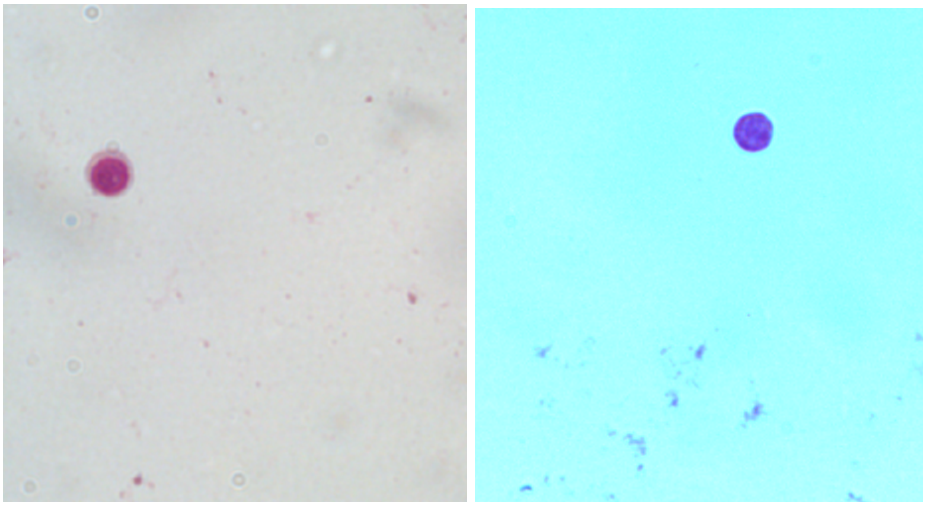


Figure 47. Microscopic image (x40) of a sorted CD4+ cell (A) and a sorted ILC2 (B).

Discussion

To our knowledge this is the first report of IL-4 and IL-13 producing ILC2s in cord blood. Cord blood from boys and girls differed significantly in the amount of ILC2s, whereby they were more abundant in cord blood from boys. ILC2 number was very small but interestingly, compared to CD4+ cells, a higher proportion of ILC2s (36.9% SD:23.2% vs 0.27% SD:0.11%) seemed to be ready and able to produce IL-13. When ILC2s were cultured with Budesonide they seemed more steroid sensitive than CD4+ cells. In cord blood all sub-groups of ILCs were detectable with a predominance of ILC1s.

The first paper reporting ILCs in cord blood was the paper by Forsberg [50]. Similar to our study ILC2s were defined as Lin-CD56-CD161+CD127+CRTH2+, were found in cord blood in comparable proportions (about 0.1% of lymphocytes) and in greater quantity in boys.

Before adolescence asthma prevalence is higher in males than in females. Only during puberty, likely because of an androgen-mediated inhibition of type 2 inflammation, this trend is reversed with asthma and most allergic disorders become more frequent in females [54]. The higher numbers of ILC2s found in baby boys supports the proposition that the higher prevalence of atopic disease in boys during childhood may be present from birth. However, Forsberg et al. followed-up the children until 6 years of age and did not detect any difference in terms of allergy development in those who had a greater number of ILC2s in cord blood [50]. Cord blood ILC2s might not have been related to allergy development for different reasons: 1) the higher number of ILC2s found in newborn boys can be an intrinsic factor predisposing to allergy development but extrinsic factors like allergen exposure may be essential to recruit and activate these cells; 2) the highest levels of ILC2s reported in asthmatics have been primarily observed at mucosal surfaces rather than systemically as demonstration of an exposure-driven recruitment and activation; 3) cord blood ILC2s may be just the natural consequence of the physiological Th2 activation seen during pregnancy and they might not be implicated in newborn allergy development; 4) neonatal immune system is physiologically skewed towards type 2 immunity and therefore ILC2s may behave in this context like naive cells of the innate immune system.

The presence of ILC2s in human neonatal lung has not been assessed yet. However, recent evidence from murine models suggests that the process of birth and particularly the first breath and the exposure to external environment is enough to trigger IL-33 production from alveolar epithelial cells and therefore activate the ILC2 pathway, particularly IL-13 secretion [55, 56]. Our data confirm that ILCs are present and active from birth in human cord blood and have a strong potential of producing cytokines, especially

IL-13. However, it is unknown whether ILC2s are also present and active in the newborn lung from birth or whether they migrate from blood to effector sites only after specific stimuli.

It has been suggested that the higher GATA-3 expression found in cord blood ILC2s compared to adults might have signified a greater activity and cytokine-producing capacity [50]. We assessed ILCs cytokine production and observed that the cytokine-producing capacity of ILC2s is actually high. Although CD4+ cells were greatly more abundant than ILC2s (54.19% SD:8.96 vs 0.015% SD:0.012), less than 1% of CD4+ cells had the capacity to secrete IL-13 while up to 76% of ILC2s (considered as percentage of Lin-CD127+CD56-CD161+CRTH2+GATA3+ cells) seemed to be ready to release this type 2 cytokine. This finding might be due to the fact that ILC2s being part of the innate immune system are ready to release interleukins even in the naive context of a newborn while CD4+ cells may need a stimulus to be activated. Therefore, newborn ILC2s may be able to rapidly develop Th2 responses even in absence of CD4+ cells activation. Although memory Th2 cells have also been found to produce IL-13 after stimulation with epithelial cytokines IL-33 and IL-25 [57] [58], during the neonatal period no evidence of this pathway has been found in mouse models so far, maybe because adaptive T cell immunity becomes fully competent only later in life [56].

In the neonatal period ILC2s may play an essential role in immune homeostasis and in type 2 responses, and may mediate interactions between innate and adaptive type 2 immune pathways. The neonatal period is a critical window for immune development and the events and the exposures occurring in early life can have a long-term impact on immune-mediated diseases like allergy and asthma [59].

Considering the previous findings on the increased levels of IL-33 in STRA patients [26], we decided to perform sorting and culture assays with ILC2s to investigate the behaviour of ILC2s with steroids. Our preliminary results of ILC2s cultures with budesonide suggest that ILC2s are steroid sensitive and even more susceptible to death than CD4+ cells. The response to steroids of ILC2s has been investigated by a few studies with conflicting results. Firstly, ILC2 resistance to steroids was reported in a mouse model of allergic airway inflammation after stimulation with IL-7 and TSLP and associated with STAT5 phosphorylation [60]. Very recently this finding was confirmed also in ILC2s from BAL taken from adult patients with refractory asthma suggesting that the crucial mechanism leading to steroid resistance is the up-regulation of IL-7Ra induced by IL-7 and TLSP [61]. Similarly, in a mouse model of nasal allergic rhinitis the exposure to dexamethasone reduced Th2 cells inflammation but not ILC2 activation suggesting steroid resistance of these cells [62]. Conversely, in two other studies steroid treatment attenuated the production of IL-5 and IL-13 by ILC2s after IL-33 stimulation [63] and increased their apoptosis both in vivo and in vitro [64]. More studies are needed to clarify weather or not ILC2s are

resistant to steroids in patients with allergic asthma.

We are aware that this study has limitations. To compare the actual production of IL-4 and IL-13 by CD4+ cells and ILC2s, quantitative measurements should have been undertaken. Gender difference in ILC2 proportions should be investigated not only in a greater number of children but also in newborn serum and eventually in the airways at different time points after birth. These results may clarify the role of ILC2s during the first weeks of life and track their pathway from cord blood to the effector sites.

The results obtained from the experiments made on ILC2s cultures have to be confirmed because very few ILC2s were sorted and recovered. In addition these cells, maybe because of the sorting process, showed poor viability in culture. The effect of budesonide should be tested at lower concentrations because the scarcity of ILC2s may have affected their mortality. Immortalized cell lines of ILC2s might be a good option to better assess functional experiments and resistance to steroids.

References

1. Mjosberg, J.M., et al., *Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161.* Nat Immunol, 2011. **12**(11): p. 1055-62.

2. Spits, H. and T. Cupedo, *Innate lymphoid cells: emerging insights in development, lineage relationships, and function.* Annu Rev Immunol, 2012. **30**: p. 647-75.

3. Moro, K., et al., *Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells.* Nature, 2010. **463**(7280): p. 540-4.

4. Yokota, Y., et al., *Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2.* Nature, 1999. **397**(6721): p. 702-6.

5. Annunziato, F., C. Romagnani, and S. Romagnani, *The 3 major types of innate and adaptive cell-mediated effector immunity.* J Allergy Clin Immunol., 2015. **135**(3): p. 626-35.

6. Diefenbach, A., M. Colonna, and S. Koyasu, *Development, differentiation, and diversity of innate lymphoid cells.* Immunity, 2014. **41**(3): p. 354-365.

7. Spits, H., et al., *Innate lymphoid cells--a proposal for uniform nomenclature.* Nat Rev Immunol, 2013. **13**(2): p. 145-9.

8. Barlow, J.L. and A.N. McKenzie, *Type-2 innate lymphoid cells in human allergic disease.* Curr Opin Allergy Clin Immunol, 2014. **14**(5): p. 397-403.

9. Neill, D.R., et al., *Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity.* Nature, 2010. **464**(**7293**): p. 1367-70.

10. Halim, T.Y., et al., *Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation.* Immunity, 2012. **36**(3): p. 451-63.

11. Wong, S.H., et al., *Transcription factor RORalpha is critical for nuocyte development.* Nat Immunol, 2012. **13**(3): p. 229-36.

12. Kim, B.S., D. Wojno Ed Fau - Artis, and D. Artis, *Innate lymphoid cells and allergic inflammation.* Curr Opin Immunol, 2013. **25**(6): p. 738-44.

13. McKenzie, A.N., *Type-2 innate lymphoid cells in asthma and allergy.* Ann Am Thorac Soc, 2014. **11**(Suppl. 5): p. S263-70.

14. Huntington, N.D., et al., *Innate lymphoid cells: parallel checkpoints and coordinate interactions with T cells.* Curr Opin Immunol, 2016. **38**(1879-0372 (Electronic)): p. 86-93.

15. Levine, S.J. and S.E. Wenzel, *Narrative review: the role of Th2 immune pathway modulation in the treatment of severe asthma and its phenotypes.* Ann Intern Med, 2010. **152**(4): p. 232-7.

16. Gould, H.J. and B.J. Sutton, *IgE in allergy and asthma today.* Nat Rev Immunol, 2008. **8**(3): p. 205-17.

17. Rosenberg, H.F., P.S. Dyer Kd Fau - Foster, and P.S. Foster, *Eosinophils: changing perspectives in health and disease.* Nat Rev Immunol, 2013. **13**(1): p. 9-22.

18. Doherty, T. and D. Broide, *Cytokines and growth factors in airway remodeling in asthma.* Curr Opin Immunol, 2007. **19**(6): p. 676-80.

19. De Boever, E.H., et al., *Efficacy and safety of an anti-IL-13 mAb in patients with severe asthma: a randomized trial.* J Allergy Clin Immunol, 2014. **133**(4): p. 989-96.

20. Bossley, C.J., et al., *Pediatric severe asthma is characterized by eosinophilia and remodeling without T(H)2 cytokines.* J Allergy Clin Immunol, 2012. **129**(4): p. 974-82.

21. Bracken, M., et al., *The importance of nurse-led home visits in the assessment of children with problematic asthma.* Arch Dis Child, 2009. **94**(10): p. 780-4.

22. Bush, A. and S. Saglani, *Management of severe asthma in children.* Lancet, 2010. **376**(9743): p. 814-25.

23. Chung, K.F., et al., *International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma.* Eur Respir J, 2014. **43**(2): p. 343-73.

24. Bush, A. and L. Fleming, *Phenotypes of refractory/severe asthma.* Paediatr Respir Rev, 2011. **12**(3): p. 177-81.

25. Bush, A., et al., *Severe childhood asthma: a common international approach?* Lancet, 2008. **372**(9643): p. 1019-21.

26. Saglani, S., et al., *IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma.* J Allergy Clin Immunol, 2013. **132**(3): p. 676-685 e13.

27. Saglani, S., et al., *Interleukin-33 promotes airway remodelling in paediatric severe steroid resistant asthma.* The Journal of allergy and clinical immunology, 2013. **132**(3): p. 676-685.e13.

28. Mjosberg, J. and H. Spits, *Type 2 innate lymphoid cells-new members of the "type 2 franchise" that mediate allergic airway inflammation.* Eur J Immunol, 2012. **42**(5): p. 1093-6.

29. Lloyd, C.M. and S. Saglani, *Epithelial cytokines and pulmonary allergic inflammation.* Curr Opin Immunol, 2015. **34**: p. 52-8.

30. Castanhinha, S., et al., *Pediatric severe asthma with fungal sensitization is mediated by steroid-resistant IL-33.* J Allergy Clin Immunol, 2015. **136**(2): p. 312-22 e7.

31. Doherty, T.A., et al., *STAT6 regulates natural helper cell proliferation during lung inflammation initiated by Alternaria.* Am J Physiol Lung Cell Mol Physiol, 2012. **303**(7): p. L577-88.

32. Beale, J., et al., *Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation.* Sci Transl Med, 2014. **6**(256): p. 256.

33. Lee, H.C., et al., *Thymic stromal lymphopoietin is induced by respiratory syncytial virus-infected airway epithelial cells and promotes a type 2 response to infection.* J Allergy Clin Immunol, 2012. **130**(5): p. 1187-1196.e5.

34. Jackson, D.J., et al., *IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo.* Am J Respir Crit Care Med, 2014. **190**(12): p. 1373-82.

35. Hong, J.Y., et al., *Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness through IL-25 and type 2 innate lymphoid cells.* J Allergy Clin Immunol, 2014. **134**(2): p. 429-39.

36. Walker, J.A. and A. McKenzie, *Innate lymphoid cells in the airways.* Eur J Immunol, 2012. **42**(6): p. 1368-74.

37. Kim, H.Y., et al., *Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity.* J Allergy Clin Immunol, 2012. **129**(1): p. 216-27.e1-6.

38. Chang, Y.J., et al., *Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity.* Nat Immunol, 2011. **12**(7): p. 631-8.

39. Spits, H. and J.P. Di Santo, *The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling.* Nat Immunol, 2011. **12**(1): p. 21-7.

40. Barlow, J.L., et al., *IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction.* J Allergy Clin Immunol, 2013. **132**(4): p. 933-41.

41. Bartemes, K.R., et al., *Enhanced innate type 2 immune response in peripheral blood from patients with asthma.* J Allergy Clin Immunol, 2014. **134**(3): p. 671-678.

42. Wojno, E.D., et al., *The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung.* Mucosal Immunol, 2015. **8**(6): p. 1313-23.

43. Yu, Q.N., et al., *Increased Group 2 Innate Lymphoid Cells Are Correlated with Eosinophilic Granulocytes in Patients with Allergic Airway Inflammation. LID - 10.1159/000488050 [doi].* Int Arch Allergy Immunol 2018.

44. Allakhverdi, Z., et al., *CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation.* J Allergy Clin Immunol, 2009. **123**(2): p. 472-8.

45. Christianson, C.A., et al., *Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33.* J Allergy Clin Immunol, 2015. **136**(1): p. 59-68.

46. Ying, S., et al., *Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity.* J Immunol, 2005. **174**(12): p. 8183-90.

47. Monticelli, L.A., et al., *Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus.* Nat Immunol, 2011. **12**(1): p. 1045-54.

48. Mjosberg, J., et al., *The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells.* Immunity, 2012. **37**(4): p. 649-59.

49. Shaw, J.L., et al., *IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps.* Am J Respir Crit Care Med, 2013. **188**(4): p. 432-9.

50. Forsberg, A., et al., *GATA binding protein 3(+) group 2 innate lymphoid cells are present in cord blood and in higher proportions in male than in female neonates.* J Allergy Clin Immunol., 2014. **134**(1): p. 228-30.

51. Nagakumar, P., et al., *Type 2 innate lymphoid cells in induced sputum from children with severe asthma.* J Allergy Clin Immunol, 2016(1097-6825 (Electronic)).

52. Roediger, B., et al., *IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation.* J Allergy Clin Immunol, 2015. **136**(6): p. 1653-1663.e7.

53. Hazenberg, M.D. and H. Spits, *Human innate lymphoid cells.* Blood, 2014. **124**(5): p. 700-9.

54. Laffont, S., E. Blanquart, and J.C. Guery, *Sex Differences in Asthma: A Key Role of Androgen-Signaling in Group 2 Innate Lymphoid Cells.* Front Immunol, 2017. **8**: p. 1069.

55. Saluzzo, S., et al., *First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment.* Cell Rep, 2017. **18**(8): p. 1893-1905.

56. de Kleer, I.M., et al., *Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung.* Immunity, 2016. **45**(6): p. 1285-1298.

57. Endo, Y., et al., *The interleukin-33-p38 kinase axis confers memory T helper 2 cell pathogenicity in the airway.* Immunity, 2015. **42**(2): p. 294-308.

58. Guo, L., et al., *Innate immunological function of TH2 cells in vivo.* Nat Immunol, 2015. **16**(10): p. 1051-9.

59. Lloyd, C.A.-O. and S.A.-O. Saglani, *Development of allergic immunity in early life.* Immunol Rev, 2017. **278**(1): p. 101-115.

60. Kabata, H., et al., *Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation.* Nat Commun, 2013. **4**: p. 2675.

61. Liu, S., et al., *Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: The role of thymic stromal lymphopoietin.* J Allergy Clin Immunol, 2018. **141**(1): p. 257-268.e6.

62. Morikawa, T., et al., *Activation of group 2 innate lymphoid cells exacerbates and confers corticosteroid resistance to mouse nasal type 2 inflammation.* Int Immunol, 2017. **29**(5): p. 221-233.

63. Chen, R., et al., *Allergen-induced Increases in Sputum Levels of Group 2 Innate Lymphoid Cells in Subjects with Asthma.* Am J Respir Crit Care Med, 2017. **196**(6): p. 700-712.

64. Walford, H.H., et al., *Increased ILC2s in the eosinophilic nasal polyp endotype are associated with corticosteroid responsiveness.* Clin Immunol 2014. **155**(1): p. 126-35.

Project 2 – Neonatal Intensive Care Unit, University of Parma, Parma (Italy)

Oxidative stress and ADMA in exhaled breath condensate of intubated preterm children: preliminary data

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Prof.ssa Cinzia Magnani, Direttore UOC Terapia Intensiva Neonatale Az.Ospedaliero-Universitaria di Parma

Abstract

Oxidative stress, defined as an imbalance between antioxidant capacity and reactive oxygen species (ROS) generation, may play an important role in many diseases of prematurity. Respiratory distress syndrome (RDS) and eventually bronchopulmonary dysplasia (BPD) and pulmonary hypertension (PH), some of the most common complications of preterm newborns, may be mediated by the increased production of ROS. Furthermore, PH development may also depend on the reduced synthesis of airway nitric oxide (NO) caused by the endogenous inhibitor of NO endothelial synthase asymmetric dimethyl arginine (ADMA). To date, most of the studies performed in preterms have evaluated oxidative stress biomarkers and ADMA in peripheral sites such as cord blood or blood serum while in the airways a few has investigated oxidative stress and none ADMA. The purpose of the study was to validate exhaled breath condensate (EBC) collection in ventilated preterm babies at birth and in the first days of life with the aim of assessing oxidative stress biomarkers and ADMA values in their airways. EBC is a quick and non-invasive technique that can contribute in the biochemical and molecular understanding of some cellular processes occurring in the respiratory system. EBC biomarkers might help the clinicians to estimate the severity of pulmonary pathology and the risk of BPD and PH in children born preterm.

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Background

Prematurity and oxidative stress

Preterm birth, defined as birth occurring at less than 37 weeks of gestation, has been reported to range from 5% to 10% of live births depending on the geographical area with 1% of all infants having a birth weight <1500 g (Very Low Birth Weight, VLBW) [65]. Preterm birth is associated with a broad spectrum of respiratory symptoms and lung function abnormalities, starting early in life and possibly lasting into adulthood as demonstration of the stabilised structural damage of prematurity [66]. The most severe respiratory complication of prematurity is bronchopulmonary dysplasia (BPD), a multifactorial disease mainly caused by hyperoxia, riperfusion and ventilator-associated injury and characterised by disrupted pulmonary growth with reduction in airway calibre and early airway remodelling [67]. These early damages may be mediated by the increased production of reactive oxygen species (ROS) generated by: 1) the transition from an intrauterine hypoxic environment to an extrauterine normoxic environment with a four-to five-fold higher oxygen tension; 2) mechanical ventilation; 3) ischemia-reperfusion conditions; 4) inflammatory and infective complications [68]. High levels of ROS result in a high load of oxidative stress. Oxidative stress particularly affects preterm infant due to the physiological immaturity of antioxidant defences [69] and it is implicated in the pathogenesis of several conditions of the preterm newborn, commonly referred to as "oxygen radical diseases of neonatology". Production of ROS determines the oxidation of cell macromolecules, e.g. lipids, DNA and proteins, leading to a variety of products including alkanes, aldehydes, oxidated nucleotides and oxidated amino acids [70]. Among the mechanisms of free radical damage, lipid peroxidation is probably the most extensively investigated process. ROS oxidation of cell membrane phospholipids produces chain reactions whose targets are the (poly)unsaturated fatty acids (P)UFA and results in the formation of unstable lipid hydroperoxides and of secondary carbonyl compounds such as aldehydic products [71]. Among the products of lipid peroxidation, malondialdehyde (MDA) and 8-isoprostane are some of the most reliable biomarkers of oxidative stress in vivo [72, 73]. Besides MDA, some a,b-unsaturated aldehydes, namely, 4-hydroxyhexenal (4-HHE) and 4-hydroxynonenal (4-HNE), are known to be formed by peroxidation of o-3 and o-6 PUFAs, respectively [71]. To neutralise the overproduction of ROS the organism develops enzymatic antioxidant defences like superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) and non-enzymatic defences like vitamins E and A, and glutathione [74]. Therefore, supplementation with enzymatic and non-enzymatic antioxidants might have beneficial effects in decreasing injury from excess production of ROS [75]. Since neonates typically present lower levels of plasma antioxidants, in our clinical practice vitamin E is

supplemented at birth in all preterm infants born with a VLBW to reduce the risk of some of the oxygen radical diseases such as intracranial haemorrhage and severe retinopathy [76]. On the other hand, to contrast the injury process of BPD, which has been hypothesized to begin as acute inflammatory changes secondary to toxic free oxygen radicals [77], preterm resuscitation is performed with low FiO_2 concentrations according to the most recent evidence [78].

Oxidative stress measurement in preterm newborns

Oxidative stress has been consistently associated with lung injury early in life in infants who progress towards BPD [79]. Urinary (o-tyrosine, 8-oxo-dihydroxyguanosine, 8-isoprostane) and blood (oxidized glutathione, MDA, 8-isoprostane) markers of oxidative stress were found to be increased in preterm infants resuscitated at birth with high FiO_2 concentrations [80, 81] and associated to increased risk of BPD (Vento M 2009) [82]. Conversely, surfactant administration has been proven to lower oxidant stress as shown by reduced levels of oxidized glutathione in favour of reduced glutathione in tracheal aspirates soon after the instillation of surfactant [83]. Exogenous surfactant actually contains SOD and CAT [84] as demonstrated in a rabbit model by the increase of SOD content in alveolar type II cells after surfactant administration [85].

However, considering the increasing interest in oxidative stress as one of the determinants of lung injury early in life, airway oxidative stress has been assessed only in a few studies in preterm children [86-90]. Most of the research explored oxidative stress markers in cord blood or serum and constantly reported increased levels of 8-isoprostane [91] and MDA [80, 92, 93] in children born preterm compared to children born at term. Higher values of oxidative stress markers inversely correlated with gestational age (GA) [91, 93] and were associated to a greater risk of BPD development [79, 91, 94, 95]. The few studies involving airway samples gave similar or inconsistent results. Bronchoalveolar lavage (BAL) analysis during the first week of life showed that preterm children exposed to oxygen therapy and at risk of BPD development had higher levels of MDA and decreased levels of reduced glutathione [87, 88]. Nevertheless, a study of 73 preterm infants reported that MDA levels in tracheal aspirates at day 1 and 3 of life were not predictors of BPD development [86]. In exhaled breath condensate (EBC) of intubated preterm neonates hydrogen peroxide was found increased in subjects at risk of BPD [96]. Overall, these data support the hypothesis that oxidative stress may play an important role in the pathogenesis of preterm lung disease.

Pulmonary hypertension and asymmetric dimethylarginine (ADMA)

Pulmonary hypertension (PH) is a severe complication of BPD with a prevalence estimated of 10% in

newborns with respiratory distress. PH is characterized by right-to-left extrapulmonary shunting through the foramen ovale and ductus arteriosus with resultant hypoxic respiratory failure. This condition might be the result of abnormal vasculature development in the preterm lung where hypoxic respiratory distress causes vasoconstriction of the pulmonary vasculature and higher pulmonary arterial pressures [97]. PH pathogenesis is still unclear but it may depend on the reduced synthesis of nitric oxide (NO) from the endothelium [98]. In the airways NO is synthesized in macrophages, neutrophils and endothelial and epithelial cells from L-arginine mainly by the inducible isoform of NO synthase (iNOS). NO maintains normal low pulmonary vasculature resistance and its deficiency may be caused by: down-regulation of iNOS [99], reduction of the substrate L-arginine [100], increase of arginase activity [101] or iNOS inhibition by the endogenous inhibitor asymmetric dimethylarginine (ADMA), a derivate of methylated arginine[102]. Methylarginine metabolism and ADMA are shown in Figure 1.

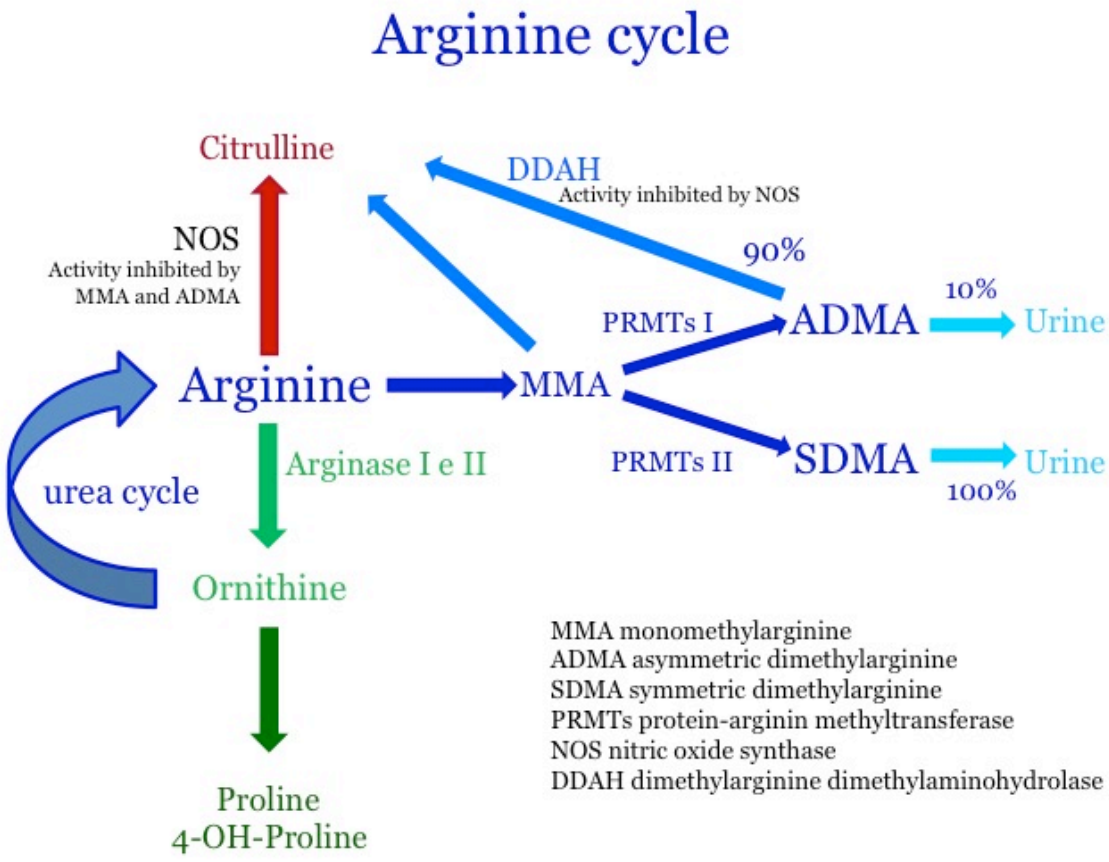


Figure 1. Asymmetric dimethylarginine (ADMA) in the arginine cycle. Free L-arginine can be metabolized by arginases to L-ornithine and urea or by nitric oxide synthases (NOS) to NO and L-citrulline. Monomethylarginine (MMA) and ADMA, but not symmetric dimethylarginine (SDMA) can be

converted to L-citrulline and mono- or diamines by a class of intracellular enzymes called dimethylarginine dimethylaminohydrolases (DDAH). MMA and ADMA, but not SDMA, act as potent endogenous inhibitors of NOS enzymes.

Arginine metilation by arginine methyltransferases (PRMT) generates protein-incorporated monomethylarginine (L-MMA), symmetric dimethylarginine (SDMA) or asymmetric dimethylarginine (ADMA). Similarly to ADMA, increased levels of SDMA result in reduced synthesis of NO; this effect is mediated by the inhibition of the cellular intake of L-arginine [103].
ADMA has been suggested as playing a role in endothelial dysfunction and PH [104] and has been found increased in the EBC of children with asthma [105]. Normally, the balance between production of ADMA and its degradation by dimethylarginine dimethylaminohydrolase (DDAH) results in low levels of ADMA and relatively little inhibition of NOS [103]. However, oxidative stress increases ADMA concentration by enhancing the activity of arginine methylating enzymes and by reducing its hydrolysis through the inhibition of DDAH [106]. Currently, little is known regarding the role of ADMA in neonatal disease. Increased levels of ADMA were found in blood serum of preterm babies with surfactant requirement at birth and at 28 days of age in those who developed BPD [107], especially if they had associated PH [108] suggesting that this molecule might be implicated in lung development and vascular resistance. ADMA values have been measured also in cord blood of children born preterm and related to the number of days of mechanical ventilation [109]. As such, lung damage due to mechanical ventilation may lead to increased ADMA release via enhanced proteolysis and cell death. If elevated serum/alveolar/pulmonary ADMA levels are associated to lung remodeling is still unknown. To date no reports on ADMA measurement in EBC of preterm children have been published. Airway biomarkers can be a potentially relevant indicator of ongoing airway disease and predictors of certain disease development [110]. At present there are no established markers of lung disease activity in the newborn and in the neonatal intensive care units (NICU) airway investigation mainly rely on invasive methods such as BAL or tracheal aspirate [111].

Exhaled breath condensate (EBC)

A possible non-invasive method to describe airway condition is the analysis of EBC. Exhaled gas composition depends on the oxidative stress generated in the lungs and on inflammatory and infectious responses. This approach has a number of potential major advantages: samples are easily available using non-invasive procedures, the collection is simple, cheap and can be performed repeatedly during the course of the disease process. EBC can be collected during spontaneous tidal breathing in collaborative patients

who breath from 10 to 30 min in specific mouthpiece connected to a cooled (0-4° C) collecting device or in intubated and ventilated patients through the respiratory circuit. Vapour and aerosolized droplets emerging with the breath are condensed and collected in a container that can be a Falcon tube or a specifically container designed to collect the condensate of the respiratory circuit. EBC is usually stored at −70 °C and it can later be accessed for the detection of specific mediators. All non-volatile compounds found in EBC originate in the airway lining fluid (ALF) or are reaction products of volatiles that enter EBC from the gas phase. Several volatile and non-volatile chemicals, including proteins, lipids, oxidants, and nucleotides have now been detected in EBC [112].

Guidelines on EBC measurement were published by the American Thoracic Society (ATS) in 2005 and in 2017 [113, 114]. Although in children standardized criteria are still lacking [115], the clinical application of EBC in collaborative children has been established by several studies mainly performed in asthma and cystic fibrosis [105, 116]. Interestingly, in the only study performed in adolescents born preterm EBC showed higher levels of oxidative stress regardless a history of BPD, as evidence of an ongoing airway disease [117]. A limited number of reports have been published on EBC in intubated infants and most of them measured just a limited number of molecules. In two studies the measurement of hydrogen peroxide in EBC of intubated neonates was validated and proposed as a biomarker for BPD risk development in subjects with RDS [96] [90]; in the research performed by Rosso et al. the measurement of glutathione status was suggested to monitor antioxidant capacity in intubated preterm newborns [89]. In a recent paper by a Russian group 119 proteins and 164 metabolites were found in the EBC of intubated neonates with congenital pneumonia or congenital diaphragmatic hernia and proposed as potential biomarkers for airway disease but with no mention of the most common oxidative stress molecules [118].

Hypothesis

EBC collection in ventilated preterm babies is feasible and can provide information on airways oxidative stress biomarkers and ADMA levels that can help the clinician to better understand some of the cellular processes occurring in the lung of preterm newborns.

Objectives

1. to find a non-invasive device associated to the respiratory circuit to collect EBC in ventilated children born preterm (consideration of a home-made collecting device);
2. to make sure that EBC collection does not interfere with respiratory mechanics monitoring patient's parameters (systolic and diastolic pressure, heart rate and oxygen saturation) throughout the study;
3. to assess the modality by which EBC can be collected in ventilated preterm children including duration of the collection, ambient conditions, amount of fluid obtained, conservation of the sample;
4. to use the validated method of measuring oxidative stress biomarkers, ADMA and aminoacids related to arginine cycle in EBC by liquid chromatography tandem-mass spectrometry technique;
5. to assess the trend of oxidative stress markers and ADMA levels over time in children born preterm who undergo invasive or non-invasive ventilation for RDS;
6. to correlate the levels of oxidative stress markers and ADMA with: a) days of oxygen therapy; b) days of ventilation; c) BPD and PH development.

Aims

We aimed to:

1. evaluate the feasibility of collecting EBC in intubated preterms through a collecting device connected to the respiratory circuit;
2. assess oxidative stress biomarkers and ADMA values (with related aminoacids) in EBC of preterm children with RDS who underwent invasive or non-invasive ventilation and oxygen therapy;
3. evaluate if EBC biomarkers could estimate lung disease and the risk of development of BPD and/or PH.

Study design

Prospective study to assess the feasibility of EBC collection in ventilated preterm newborns and EBC biomarkers measurement.

Methods

Between January 2017 and April 2018 we recruited children born preterm (GA <32 wks) admitted to the NICU of the Paediatrics Department of the University of Parma (Parma, Italy) who underwent invasive or non-invasive ventilation due to respiratory distress. We aimed to recruit as controls children born at term ventilated for surgical interventions.

Exclusion criteria were infant hypoxic ischemic encephalopathy, cranial malformations, metabolic disease, congenital heart disease and pulmonary congenital malformations.

Demographic data (mode of delivery, GA, intrauterine growth restriction and/or born small for gestational age, Apgar score, birth weight, sex, number of days of oxygen therapy, number of days of invasive and not invasive ventilation, BPD diagnosis, pharmacological or surgical closure of patent ductus arteriosus) were recorded. According to GA children were classified as extremely preterm (<28 wks of GA) or very preterm (28-<32 wks GA) and according to birth weight (BW) as Low Birth Weight (BW <2500 g), Very Low Birth Weight (BW <1500 g) or Extremely Low Birth Weight (BW <1000 g). Intrauterine growth restriction (IUGR) was defined according to the obstetrical records reported during the gestation and small for gestational age (SGA) was defined when BW was $<10^{th}$ percentile for GA plotted on standardized fetal-infant growth curves [119]. Signs of perinatal depression were assessed as: low Apgar scores at 1-and 5-minutes, defined as <3 and <5, respectively, and low cord pH or initial arterial infant pH less than 7.2 [120, 121]. BPD was defined as oxygen requirement for at least 28 days, plus assessment of disease severity as mild, moderate or severe according to the need for supplemental oxygen at 36 weeks post-menstrual age or at 56 days of life [122]. Systolic and diastolic pressure, heart rate and oxygen saturation were continuously monitored throughout the study period. Tidal volume (V_T), respiratory rate (RR), inspiratory to expiratory ratio (I:E) and FiO_2 were obtained from the ventilator and recorded. Partial pressure of arterial oxygen (O_2) (PaO_2), partial pressure of carbon dioxide ($PaCO_2$), pH and lactates were assessed daily with arterial blood analysis. Patients were ventilated by the disposable heated breathing circuit Ventstar (Drager®, Italia). The ventilator settings were those set by the NICU attending physicians. Temperature and humidification were maintained constant throughout the study protocol by means of an active humidifier (Fisher & Paykel Healthcare, Auckland, New Zealand). The subject's blood urea nitrogen level (BUN, mg/dl) was noted in the medical record on the day samples were obtained. EBC was collected within the first 24 hour of life and then at day 1 3, 5, 7 and 15.

EBC biomarkers

In neonatal EBC we measured the following biomarkers:

- Urea. The levels of EBC biomarkers are influenced not only by their levels in lung lining fluid but also by the volume of water vapour that condenses during EBC collection. For this reason, the use of a biomarker of dilution has been recommended. Urea has been proposed and utilized as a promising dilution biomarker for EBC non-volatile biomarkers due to its even distribution throughout the body and relatively low volatility. Urea is a diffusible compound with a very limited volatility, its concentrations in EBC are unaffected by chronic airway disease and individual variation of urea in plasma are reflected by parallel variation of urea in EBC. In repeated measurements within the same individual, the use of urea as a normalizing factor has been proposed to control intra-individual variability [123].
- ADMA, SDMA and the aminoacids related to arginine cycle (arginine, ornithine, citrulline, proline) (Fig.1).
- Adenosine (Ado). Adenyl purines are released onto the airway surfaces by airway epithelial and inflammatory cells where they act as signalling molecules to regulate host defences [124, 125]. Adenosine is a purine that has been linked to inflammatory airways diseases and in particular is elevated in subjects with chronic obstructive pulmonary disease (COPD) [126], asthma and cystic fibrosis (CF) [127] [128]; it was suggested as a potential biomarker of neutrophilic airway inflammation in older children and adults with CF [127, 129].
- 4-hydroxynonenal (4-HNE). 4-HNE is an α,β-unsaturated hydroxyalkenal that is produced by lipid peroxidation in cells as a result of oxidative stress. Higher concentrations have been shown to be associated to cell apoptosis and necrosis.
- Malondialdehyde (MDA). MDA is generated by lipid peroxidation of the polyunsaturated fatty acids arachidonic and docosa-hexenoic acid. As 4-HNE, MDA is a marker of oxidative stress. MDA levels in EBC increase during asthma exacerbations but decrease after steroid treatment [112].

EBC analysis

According to a previous study performed in intubated preterms [89], we normalized the EBC values with the urea dilution factor (*f*). A dilution factor is the ratio between the same analyte in two different biological matrices. Samples were corrected for dilution by multiplication with the urea dilution factor (*f*)

defined as *f* = Patient BUN/sample urea as previously described [130].

To assess arginine bioavailability we used the concept of "global arginine bioavailability ratio" (GABR) which accounts for levels of the substrate (arginine) and its major catabolic products (ornithine and citrulline). GABR might serve as a more comprehensive concept of reduced NO synthetic capacity compared with levels of free arginine {Tang, 2009 #612}.

In EBC samples biomarkers of oxidative stress (MDA and 4-HNE), ADMA, SDMA and other related aminoacids, Ado and Urea as dilution factor were quantified by LC-MS/MS methods.

MDA and 4-HNE were determined after derivatisation by 2,4-dinitro-phenylhydrazine (DNPH) as previously described [71], with some modifications and expresses in nM (limits of detection LODs 0.05 and 0.01 nM). Briefly, the EBC samples were derivatised with an equal volume of DNPH (1.26 mM) and stored at room temperature for 2 h. After this time, 5 μL are injected into the LC–MS/MS system, which consisted of an Agilent HP 1100 series binary pump (Palo Alto, CA, USA) coupled to a AB Sciex API 4000™ triple-quadruple mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with a TurboIonSpray™ (TISP) interface. Chromatography was performed on a Atlantis®dC_{18} column (2.1 x 100 mm i.d., 3μm; Waters, Milford, MA, USA) using variable proportions of 10 mM aqueous formic acid and methanol/acetonitrile (95/5, v/v) at a flow rate of 0.2 mL/min. MDA was ionized in positive-ion mode and 4-HNE in negative-ion mode. Both aldehydes were detected in selected-reaction monitoring (SRM) mode; limits of quantification were 0.1 nM and 0.04 nM for MDA and 4-HNE, respectively.

ADMA, SDMA and other related aminoacids (arginine, citrulline, ornithine, proline), Ado and urea were determined with the same instrument setting, but under different SRM conditions and chromatographic gradient. All the compounds were ionized in positive-ion mode; the method allowed the simultaneous quantification of amino acid derivatives and dilution factors without derivatization and relying on the principle of dilution of isotopic internal standards. The LODs and the limits of quantification (LOQs) are in the range of 0.02 to 0.26 and 0.08 to 0.88 nMol/L, respectively. For the analyses, 50 μL of EBC were used for each assay.

The protocol was reviewed and approved by the Ethics Committee of the Hospital of Parma (IRB, Prot. n**.** 0024/2017) and written informed consent was obtained for all patients according to the Italian regulations.

Statistical analysis

Statistical power

On the basis of the data obtained in the previous studies [80, 82] (considering a standard deviation (SD) up to 40% to take into account the variations from normality) and assuming that about one third of children could have lung complications [82] and that oxidative stress biomarkers could increase of about 50% at day 7, we suppose that a 2 tails t-test with a number of subjects of n=36 (mild or no lung complications) and n=18 (severe lung complications) could have a power = 90% and alpha = 2.5%.

We wanted to analyse differences between groups with ANOVA in case of normal data otherwise with Friedman and Mann-Whitney tests but we did not reach a sufficient number of samples.

Results

Participant characteristics

Five preterm subjects were included in the study. The GA range was between 24+3 and 30+6 weeks (mean GA 28.5 ± 2.73 wks). Only one control subject was recruited (GA 36+2 wks, BW 2170 g, SGA, ventilated for 3 days for surgical repair of an anorectal malformation, EBC collected at day 3 and 5 of life). The distribution and characteristics of preterm infants are shown in Table 1.

Characteristics	
Male	4
Gestational age, weeks	
<28	2
28-29	0
30-31	3
Median (10^{th}-90^{th} centile)	30 (25-31)
Birth weight, g	
<1500	5
≥1500	0
Median (10^{th}-90^{th} centile)	777.5 (1030-685)
SGA (<10^{th} centile)	3
Surfactant administration	3
Mechanical ventilation	3
Days on mechanical ventilation, mean (SD)	26.7 (22.3)
Median (10^{th}-90^{th} centile)	27 (0-53)
Non-invasive ventilation	5
Days on mechanical ventilation, mean (SD)	27.5 (18.4)
Median (10^{th}-90^{th} centile)	28.5 (6-47)
RDS	3
BPD	3
Oxygen at 36 days of postmenstrual age	3
Days on oxygen therapy, mean (SD)	68 (44.7)
Median (10^{th}-90^{th} centile)	(5-115)
Patent ductus arteriosus	1
Pulmonary hypertension	1

Table 1. Characteristics of preterm children included in the study.

SGA, small for gestational age [119]; RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia, defined as supplemental oxygen dependency at 36 weeks of postmenstrual age [122].

Exhaled breath condensate collection

As previously reported by our group in a study performed in ventilated adults, we attempted to use a home-made collecting device sited in-line with the expiratory limb of the ventilator circuit {Vaschetto, 2015 #613}. The device is composed by two 9 mm tubes connected in parallel and placed in the expiratory limb next to the Y-piece that enters a 50 ml falcon conical centrifuge tube. To avoid air leaks in the respiratory circuit a round shape connector was inserted on each of the two 9 mm tubes (Fig. 2).

Figure 2. The home-made collecting device for EBC.

Due to air leaks and frequent alarm signals from the respiratory circuit, we decided to use the built-in condensate collecting device in-line with the expiratory limb of the heated Ventstar circuit (Drager®, Italia) (Fig. 3). EBC was collected at room temperature and the collecting device emptied in a sterile Falcon tube when contained at least 3 ml of condensate. The total volume was then aliquoted and stored at −80 ◦C until

assayed.

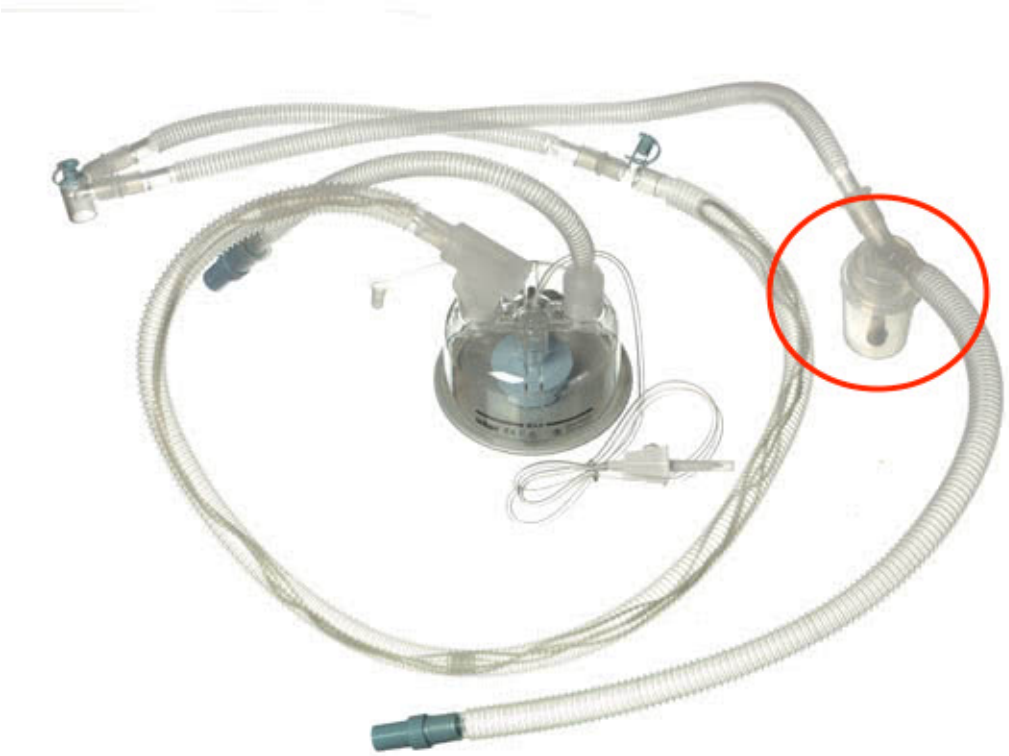


Figure 3. Ventstar neonatal heated breathing circuit (Drager®, Italia). In the red circle the container to collect the condensate.

EBC samples

All biomarkers (urea, arginine, citrulline, ornithine, proline, adenosine, ADMA, SDMA, MDA, 4-HNE) were detectable and measurable in EBC fluid. The mean production of EBC in the collecting device was 1.68 (SD:1.56) ml/hour. Mean duration of collection was 2.5 (SD:1.3) hour.

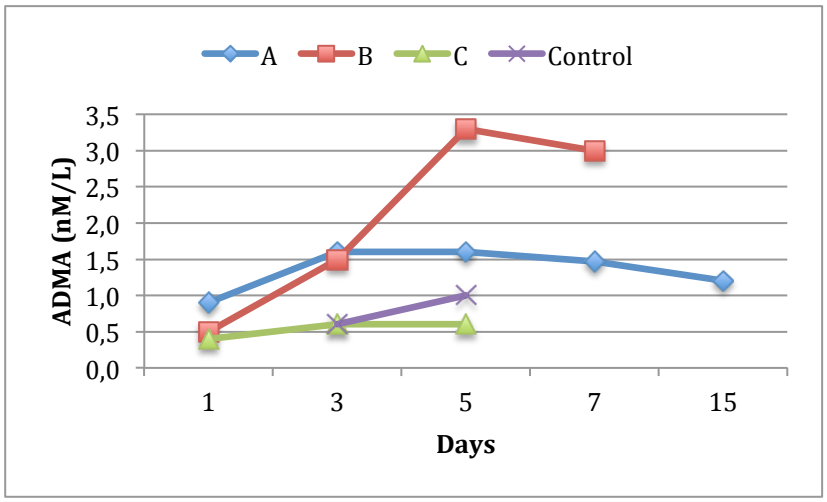
For 1 patient we collected EBC at 5 different time points (day 1, 3, 5, 7 and 15) (patient A), for 1 patient at 4 (day 1, 3, 5 and 7) (patient B), for 1 patient at 3 (day 1, 3 and 5) (patient C) and for 2 patients only at day 1 because ventilation stopped before 24th hour after delivery. The latter two patients were not considered for analysis since we had just 1 time point. Mean values of oxidative stress biomarkers, ADMA and related aminoacids measured in EBC of patients A, B and C are reported in Table 2. No complications were recorded during EBC collection.

Urea values		
Sample urea (nM/L)	80.1 (14.1)	
BUN at sample collection (mg/dl)	58.9 (39.6)	
Urea dilution factor *f*	0.74 (0.45)	
EBC biomarkers	**Raw values**	**Corrected values for *f***
Citrulline (nM/L)	16.88 (9.28)	11 (6.15)
Arginine (nM/L)	12.91 (6.29)	8.77 (6,23)
Ornithine (nM/L)	16.32 (7.56)	11.96 (8.79)
Proline (nM/L)	14.33 (6.44)	10.09 (6.83)
ADMA (nM/L)	1.92 (0.47)	1.39 (0.93)
SDMA (nM/L)	1.94 (0.38)	1.39 (0.90)
Adenosine (nM/L)	2002.41 (352.72)	1472.92 (990.61)
MDA (nM/L)	0.64 (0.25)	0.47 (0.29)
4-HNE (nM/L)	3.17 (3.27)	1.96 (2.31)

Table 2. First section: mean values (SD) of urea concentration in EBC samples and blood urea nitrogen (BUN) at time of sample collection with urea dilution factor *f*.
Second section: mean values (SD) of oxidative stress biomarkers (MDA, malondialdeyde; 4-HNE, 4-hydroxynonenal), adenosine, ADMA (asymmetric dimethylarginine), SDMA (symmetric dimethylarginine) and related aminoacids (citrulline, arginine, ornithine, proline) measured in EBC.

Due to the scarcity of cases we could not perform any statistical analysis. To date we can only observe that: 1) all EBC biomarkers tended to increase over time; 2) there might be increased values of ADMA (Fig. 4a) and an associated increase in ADMA/Arg ratio (Fig. 4b) in the patient with PH; 3) ADMA and ADMA/Arg ratio seem to have a similar trend in all patients (Fig. 5); 4) citrulline, ornithine and proline can not be related to arginine values; 5) oxidative stress markers (MDA and 4-HNE) did not show a clear regular trend over time and are not correlated (Fig. 6); 6) in all patients adenosine levels tended to slightly increase over time; 7) we found no correlations between arterial pH or FiO_2 with MDA or 4-HNE.

a)



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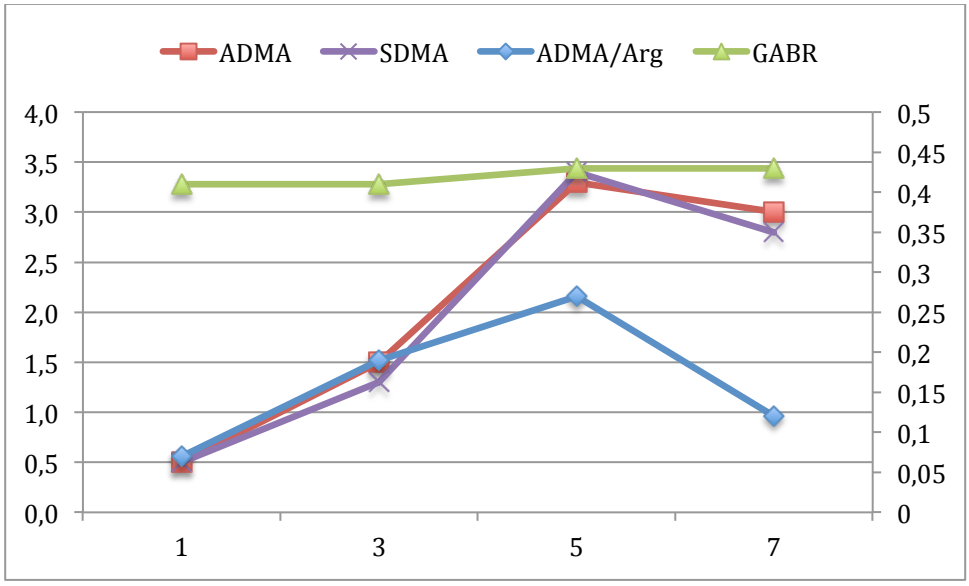


Figure 4. a) ADMA in EBC of the 3 preterm patients (A, B and C) and 1 control subject at different time points (day 1, 3, 5, 7 and 15). Patient B suffered from pulmonary hypertension.

b) ADMA, SDMA, ADMA/Arg ratio and GABR [Global arginine bioavailability ratio = arginine /(ornithine+citrulline)] trend in patient B.

ADMA and SDMA values are plotted on the left axis.

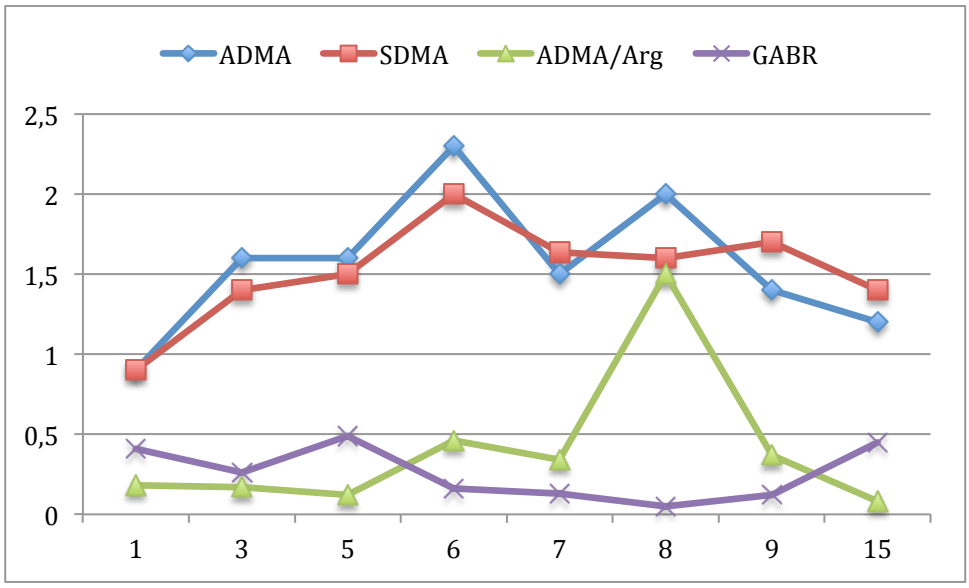
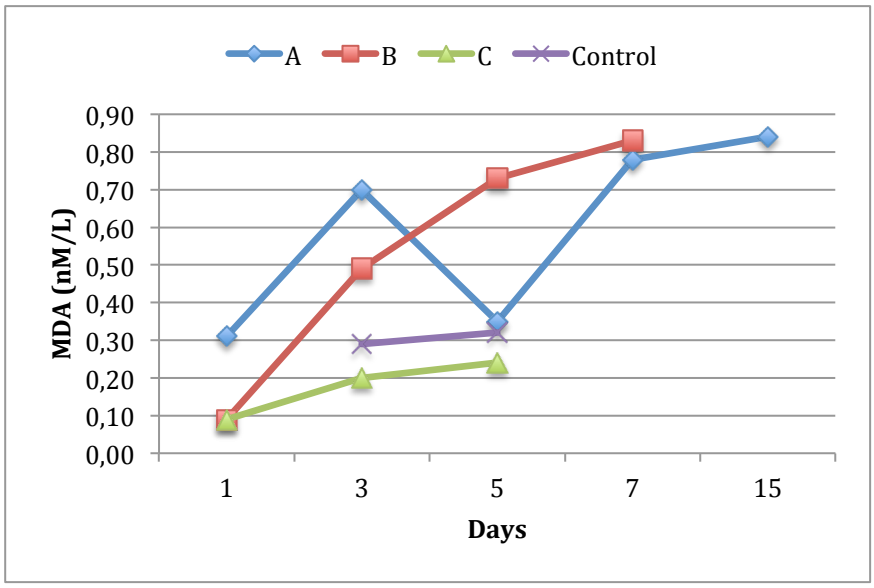


Figure 5. ADMA, SDMA, ADMA/Arg ratio and GABR [Global arginine bioavailability ratio = arginine /(ornithine+citrulline)] trend over 15 days in patient A (in this patient EBC was collected at several time points).

a)



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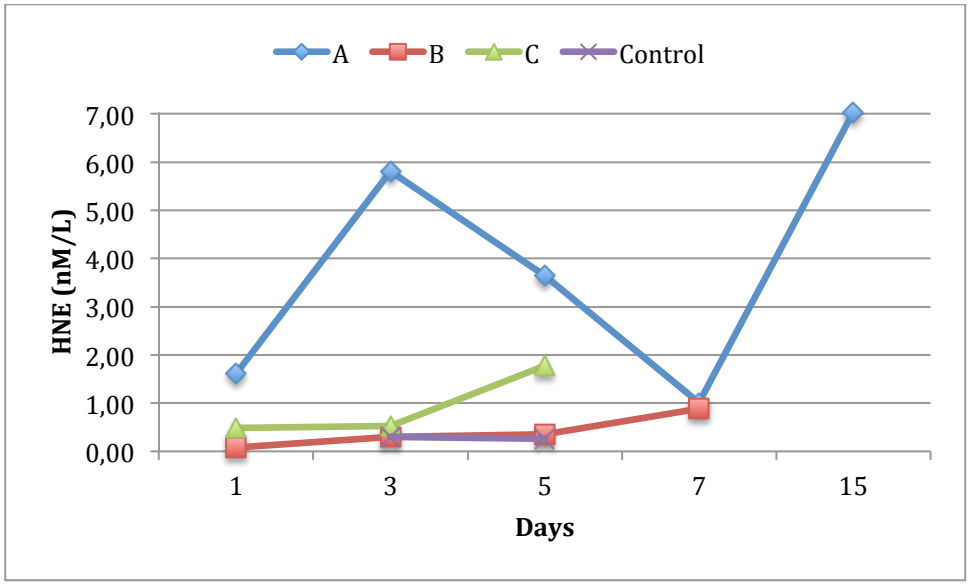

Figure 6. MDA (a) and 4-HNE (b) biomarkers in EBC of 3 preterm patients (A, B and C) and 1 control subject at different time points (day 1, 3, 5, 7 and 15).

Discussion

We showed that EBC can be collected without complications in ventilated preterm newborns and that oxidative stress biomarkers, adenosine, ADMA and aminoacids related to arginine cycle are detectable and measurable by liquid chromatography tandem-mass spectrometry.
Associations between EBC biomarkers and clinical outcomes were not possible due to the paucity of subjects recruited. Limiting our comment to a descriptive observation, we noticed that ADMA levels were doubled at day 5 and 7 in the EBC of the patient with PH compared to the other two preterm patients (who did not suffer from PH) and to control. In all patients ADMA/Arg ratio seemed to increase with the increasing of ADMA. Oxidative stress biomarkers progressively increased in all patients over time but neither a peak nor a correlation with oxygen requirements or arterial pH (as sign of respiratory/metabolic acidosis) was seen in any of the patients.

It has been described that prematurity is associated to high levels of oxidative stress and that preterm newborns have immature antioxidant defences. Any impairment of the balance between oxidative stress and antioxidative defences may durably harm the newborn because increased oxidative stress results in production of ROS, alteration of cellular metabolism and eventually cellular injury. To limit the exposure of the newborn to oxidative stress and ROS, in the neonatal period it is strongly recommended to limit the exposure to oxygen, the main cause of oxidative stress [78], and to reduce mechanical insults by limiting the pressure and the volume of gas delivered to the lungs during ventilation. So far, a biomarker capable of describing airway conditions and support the clinician in the management of a preterm newborn with respiratory distress has not been found. Considering the role of oxidative stress in the pathogenesis of many diseases of prematurity, several studies focused on markers such as 8-isoprostane or MDA as predictive of clinical outcomes. However, to date a few studies measured oxidative stress in airway samples like BAL [87, 88], tracheal aspirates [86] or EBC [89, 90, 96] and none found a clear association with BPD development suggesting that a stable oxidative stress biomarker predictive of lung complications has not been yet identified. Furthermore, the studies on EBC analysed only hydrogen peroxide [90] [96] and glutathione status [89] as markers of oxidative stress. The measurement of MDA or 4-HNE in the EBC of a consistent number of preterm subjects could demonstrate peaks in oxidative stress dependent on oxygen requirement or metabolic decompensation and eventually set reference values for this population. Considering that cord blood oxidative stress biomarkers were reported to predict BPD risk [82] [131] [78], we believe that oxidative stress measured in EBC, once associated with clinical variables, could be even a better biomarker to predict pulmonary outcome in preterm children because is not affected by maternal

conditions such as preeclampsia or chorioamnionitis [132]. Knowing oxidative stress thresholds or being aware that during the first days of life there might be a peak in such biomarkers might influence the clinical management of preterm newborns for example considering an appropriate time to give vitamin E or to repeat surfactant administration. Conversely to the EBC analysed in adults, where MDA correlates with 4-HNE [133], in our samples the two markers showed different values over time. This finding might confirm that during this early age the antioxidant system is not completely functional.

Based on the experience of our group, EBC can represent very well lung environment and can be considered a good matrix in which several biomarkers from pulmonary and systemic processes may be identified. To our knowledge, this is the first report about the measurement of ADMA in EBC of preterm newborns. ADMA was higher in a cohort of children with congenital heart disease and PH compared to children with congenital heart disease but without PH suggesting that ADMA can be implicated in PH pathogenesis through the inhibition of NOS [134]. Our preliminary data showed that starting from day 5 of life ADMA values were doubled in the preterm patient who developed PH. This might suggest that the increase of this molecule precedes the clinical manifestation of PH behaving like a biomarker of effect. However, a greater number of patients and a longer follow-up with more doses at different time points are needed to pursue this hypothesis.

The few studies on EBC in preterms confirmed that EBC collection from the exhalation limb of the ventilator could be feasible and safe in intubated newborns without interfering with airway compliance and resistance [89, 96, 135]. Furthermore, this approach reduces possible contamination from volatiles in the atmosphere or in the mouth [113].

The small sample size of this pilot study limits the interpretation of the data and an adequately powered clinical study with a much larger cohort of preterm children is needed to extend these results, estimate biomarkers trend over time and eventually assess associations with clinical outcomes during follow-up. Furthermore, the analysis of EBC in a bigger cohort of preterm newborns could define whether biomarkers are influenced by gestational age. It has been reported that lower gestational ages are associated with higher oxidative stress [91] but nothing is known on ADMA concentrations.

During the study we did not monitor temperature stability throughout sample collection since we did not use a cooling chamber to collect EBC and this may have influenced EBC volume and biomarker concentration [136]. A collecting cooling device is usually used to condensate the breathing air with the main aim of increasing the volume collected [114]. In our experiments the expiratory limb of the respiratory circuit produced a large amount of condensate in the associated collecting container without the need of a special cooling device. However, we are aware that the artificial humidification of the neonatal

respiratory circuit might have determined a certain dilution of biomarker concentrations. Another possible problem related to the collection of EBC at room temperature is the biomarker stability but it is reported that while molecules like hydrogen peroxide are sensitive to high temperatures, many others such as aminoacids are not affected [137]. A further limitation of our study is the duration of EBC collection that ranged from 30 minutes to 5 hours. We need to perform comparative experiments collecting EBC at a controlled temperature and for a set amount of time. Interestingly, despite the issues related to EBC collection in this kind of population (difficulty in using a cooling chamber connected to the collecting device, long duration of collection, humidifier associated to the respiratory circuit), we were able to detect and measure several biomarkers. Although liquid-chromatography tandem mass spectrometry is widely used in our laboratory to validate EBC analysis [71, 138], standardization of EBC interpretation remains an important issue, especially in neonates. According to a previous study performed in newborns [89], we decided to use the urea dilution factor to normalize the concentration of the biomarkers measured in EBC. However, normalization for dilution is still a matter of debate [139]. All these issues might be addressed if we achieved a standardized approach for collection and analysis of EBC in neonatal age in terms of methodology and timing.

In this study we aimed to assess the feasibility of measuring biomarkers in EBC collected from ventilated preterm children using a commercially available collection device and we demonstrated that EBC collection was well tolerated without significant changes in respiratory status. Evaluation of EBC holds potential as a non-invasive approach to monitor antioxidant status an ADMA levels in premature newborns. We are aware that there is still a long way before the analysis of EBC biomarkers becomes part of the routine clinical practice and further investigations are required to identify those infants at greatest risk for subsequent pulmonary oxidant-induced injury. Determining ADMA levels and oxidative stress status in the developing lung could advance our understanding of the mechanisms underlying antioxidant depletion and BPD development BPD in the neonate. Once obtained normal reference data, characterizing airway biomarkers may be very helpful in the clinical setting to predict pulmonary outcome and consider specific therapies.

References

1. Mjosberg, J.M., et al., *Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161.* Nat Immunol, 2011. **12**(11): p. 1055-62.

2. Spits, H. and T. Cupedo, *Innate lymphoid cells: emerging insights in development, lineage relationships, and function.* Annu Rev Immunol, 2012. **30**: p. 647-75.

3. Moro, K., et al., *Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells.* Nature, 2010. **463**(7280): p. 540-4.

4. Yokota, Y., et al., *Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2.* Nature, 1999. **397**(6721): p. 702-6.

5. Annunziato, F., C. Romagnani, and S. Romagnani, *The 3 major types of innate and adaptive cell-mediated effector immunity.* J Allergy Clin Immunol., 2015. **135**(3): p. 626-35.

6. Diefenbach, A., M. Colonna, and S. Koyasu, *Development, differentiation, and diversity of innate lymphoid cells.* Immunity, 2014. **41**(3): p. 354-365.

7. Spits, H., et al., *Innate lymphoid cells--a proposal for uniform nomenclature.* Nat Rev Immunol, 2013. **13**(2): p. 145-9.

8. Barlow, J.L. and A.N. McKenzie, *Type-2 innate lymphoid cells in human allergic disease.* Curr Opin Allergy Clin Immunol, 2014. **14**(5): p. 397-403.

9. Neill, D.R., et al., *Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity.* Nature, 2010. **464**(**7293**): p. 1367-70.

10. Halim, T.Y., et al., *Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation.* Immunity, 2012. **36**(3): p. 451-63.

11. Wong, S.H., et al., *Transcription factor RORalpha is critical for nuocyte development.* Nat Immunol, 2012. **13**(3): p. 229-36.

12. Kim, B.S., D. Wojno Ed Fau - Artis, and D. Artis, *Innate lymphoid cells and allergic inflammation.* Curr Opin Immunol, 2013. **25**(6): p. 738-44.

13. McKenzie, A.N., *Type-2 innate lymphoid cells in asthma and allergy.* Ann Am Thorac Soc, 2014. **11**(Suppl. 5): p. S263-70.

14. Huntington, N.D., et al., *Innate lymphoid cells: parallel checkpoints and coordinate interactions with T cells.* Curr Opin Immunol, 2016. **38**(1879-0372 (Electronic)): p. 86-93.

15. Levine, S.J. and S.E. Wenzel, *Narrative review: the role of Th2 immune pathway modulation in the treatment of severe asthma and its phenotypes.* Ann Intern Med, 2010. **152**(4): p. 232-7.

16. Gould, H.J. and B.J. Sutton, *IgE in allergy and asthma today.* Nat Rev Immunol, 2008. **8**(3): p. 205-17.

17. Rosenberg, H.F., P.S. Dyer Kd Fau - Foster, and P.S. Foster, *Eosinophils: changing perspectives in health and disease.* Nat Rev Immunol, 2013. **13**(1): p. 9-22.

18. Doherty, T. and D. Broide, *Cytokines and growth factors in airway remodeling in asthma.* Curr Opin Immunol, 2007. **19**(6): p. 676-80.

19. De Boever, E.H., et al., *Efficacy and safety of an anti-IL-13 mAb in patients with severe asthma: a randomized trial.* J Allergy Clin Immunol, 2014. **133**(4): p. 989-96.

20. Bossley, C.J., et al., *Pediatric severe asthma is characterized by eosinophilia and remodeling without T(H)2 cytokines.* J Allergy Clin Immunol, 2012. **129**(4): p. 974-82.

21. Bracken, M., et al., *The importance of nurse-led home visits in the assessment of children with problematic asthma.* Arch Dis Child, 2009. **94**(10): p. 780-4.

22. Bush, A. and S. Saglani, *Management of severe asthma in children.* Lancet, 2010. **376**(9743): p. 814-25.

23. Chung, K.F., et al., *International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma.* Eur Respir J, 2014. **43**(2): p. 343-73.

24. Bush, A. and L. Fleming, *Phenotypes of refractory/severe asthma.* Paediatr Respir Rev, 2011. **12**(3): p. 177-81.

25. Bush, A., et al., *Severe childhood asthma: a common international approach?* Lancet, 2008. **372**(9643): p. 1019-21.

26. Saglani, S., et al., *IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma.* J Allergy Clin Immunol, 2013. **132**(3): p. 676-685 e13.

27. Saglani, S., et al., *Interleukin-33 promotes airway remodelling in paediatric severe steroid resistant asthma.* The Journal of allergy and clinical immunology, 2013. **132**(3): p. 676-685.e13.

28. Mjosberg, J. and H. Spits, *Type 2 innate lymphoid cells-new members of the "type 2 franchise" that mediate allergic airway inflammation.* Eur J Immunol, 2012. **42**(5): p. 1093-6.

29. Lloyd, C.M. and S. Saglani, *Epithelial cytokines and pulmonary allergic inflammation.* Curr Opin Immunol, 2015. **34**: p. 52-8.

30. Castanhinha, S., et al., *Pediatric severe asthma with fungal sensitization is mediated by steroid-resistant IL-33.* J Allergy Clin Immunol, 2015. **136**(2): p. 312-22 e7.

31. Doherty, T.A., et al., *STAT6 regulates natural helper cell proliferation during lung inflammation initiated by Alternaria.* Am J Physiol Lung Cell Mol Physiol, 2012. **303**(7): p. L577-88.

32. Beale, J., et al., *Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation.* Sci Transl Med, 2014. **6**(256): p. 256.

33. Lee, H.C., et al., *Thymic stromal lymphopoietin is induced by respiratory syncytial virus-infected airway epithelial cells and promotes a type 2 response to infection.* J Allergy Clin Immunol, 2012. **130**(5): p. 1187-1196.e5.

34. Jackson, D.J., et al., *IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo.* Am J Respir Crit Care Med, 2014. **190**(12): p. 1373-82.

35. Hong, J.Y., et al., *Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness through IL-25 and type 2 innate lymphoid cells.* J Allergy Clin Immunol, 2014. **134**(2): p. 429-39.

36. Walker, J.A. and A. McKenzie, *Innate lymphoid cells in the airways.* Eur J Immunol, 2012. **42**(6): p. 1368-74.

37. Kim, H.Y., et al., *Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity.* J Allergy Clin Immunol, 2012. **129**(1): p. 216-27.e1-6.

38. Chang, Y.J., et al., *Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity.* Nat Immunol, 2011. **12**(7): p. 631-8.

39. Spits, H. and J.P. Di Santo, *The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling.* Nat Immunol, 2011. **12**(1): p. 21-7.

40. Barlow, J.L., et al., *IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction.* J Allergy Clin Immunol, 2013. **132**(4): p. 933-41.

41. Bartemes, K.R., et al., *Enhanced innate type 2 immune response in peripheral blood from patients with asthma.* J Allergy Clin Immunol, 2014. **134**(3): p. 671-678.

42. Wojno, E.D., et al., *The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung.* Mucosal Immunol, 2015. **8**(6): p. 1313-23.

43. Yu, Q.N., et al., *Increased Group 2 Innate Lymphoid Cells Are Correlated with Eosinophilic Granulocytes in Patients with Allergic Airway Inflammation. LID - 10.1159/000488050 [doi].* Int Arch Allergy Immunol 2018.

44. Allakhverdi, Z., et al., *CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation.* J Allergy Clin Immunol, 2009. **123**(2): p. 472-8.

45. Christianson, C.A., et al., *Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33.* J Allergy Clin Immunol, 2015. **136**(1): p. 59-68.

46. Ying, S., et al., *Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity.* J Immunol, 2005. **174**(12): p. 8183-90.

47. Monticelli, L.A., et al., *Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus.* Nat Immunol, 2011. **12**(1): p. 1045-54.

48. Mjosberg, J., et al., *The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells.* Immunity, 2012. **37**(4): p. 649-59.

49. Shaw, J.L., et al., *IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps.* Am J Respir Crit Care Med, 2013. **188**(4): p. 432-9.

50. Forsberg, A., et al., *GATA binding protein 3(+) group 2 innate lymphoid cells are present in cord blood and in higher proportions in male than in female neonates.* J Allergy Clin Immunol., 2014. **134**(1): p. 228-30.

51. Nagakumar, P., et al., *Type 2 innate lymphoid cells in induced sputum from children with severe asthma.* J Allergy Clin Immunol, 2016(1097-6825 (Electronic)).

52. Roediger, B., et al., *IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation.* J Allergy Clin Immunol, 2015. **136**(6): p. 1653-1663.e7.

53. Hazenberg, M.D. and H. Spits, *Human innate lymphoid cells.* Blood, 2014. **124**(5): p. 700-9.

54. Laffont, S., E. Blanquart, and J.C. Guery, *Sex Differences in Asthma: A Key Role of Androgen-Signaling in Group 2 Innate Lymphoid Cells.* Front Immunol, 2017. **8**: p. 1069.

55. Saluzzo, S., et al., *First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment.* Cell Rep, 2017. **18**(8): p. 1893-1905.

56. de Kleer, I.M., et al., *Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung.* Immunity, 2016. **45**(6): p. 1285-1298.

57. Endo, Y., et al., *The interleukin-33-p38 kinase axis confers memory T helper 2 cell pathogenicity in the airway.* Immunity, 2015. **42**(2): p. 294-308.

58. Guo, L., et al., *Innate immunological function of TH2 cells in vivo.* Nat Immunol, 2015. **16**(10): p. 1051-9.

59. Lloyd, C.A.-O. and S.A.-O. Saglani, *Development of allergic immunity in early life.* Immunol Rev, 2017. **278**(1): p. 101-115.

60. Kabata, H., et al., *Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation.* Nat Commun, 2013. **4**: p. 2675.

61. Liu, S., et al., *Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: The role of thymic stromal lymphopoietin.* J Allergy Clin Immunol, 2018. **141**(1): p. 257-268.e6.

62. Morikawa, T., et al., *Activation of group 2 innate lymphoid cells exacerbates and confers corticosteroid resistance to mouse nasal type 2 inflammation.* Int Immunol, 2017. **29**(5): p. 221-233.

63. Chen, R., et al., *Allergen-induced Increases in Sputum Levels of Group 2 Innate Lymphoid Cells in Subjects with Asthma.* Am J Respir Crit Care Med, 2017. **196**(6): p. 700-712.

64. Walford, H.H., et al., *Increased ILC2s in the eosinophilic nasal polyp endotype are associated with corticosteroid responsiveness.* Clin Immunol 2014. **155**(1): p. 126-35.

65. Howson Cp Fau - Kinney, M.V., et al., *Born too soon: preterm birth matters.* Reprod Health, 2013. **10**(1).

66. Kinsella, J.P., S.H. Greenough A Fau - Abman, and S.H. Abman, *Bronchopulmonary dysplasia.* Lancet, 2006. **367**(9520): p. 1421-31.

67. Baraldi, E. and M. Filippone, *Chronic lung disease after premature birth.* N Engl J Med, 2007. **357**(19): p. 1946-55.

68. Shoji, H. and B. Koletzko, *Oxidative stress and antioxidant protection in the perinatal period.* Curr Opin Clin Nutr Metab Care, 2007. **10**(3): p. 324-8.

69. Lavoie, J.C. and P. Chessex, *Development of glutathione synthesis and gamma-glutamyltranspeptidase activities in tissues from newborn infants.* Free Radic Biol Med, 1998. **24**(6): p. 994-1001.

70. de Zwart, L.L., et al., *Biomarkers of free radical damage applications in experimental animals and in humans.* Free Radic Biol Med, 1999. **26**(1-2): p. 202-26.

71. Andreoli, R., et al., *Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry.* Rapid Commun Mass Spectrom, 2003. **17**(7): p. 637-45.

72. Montuschi, P., G. Barnes Pj Fau - Ciabattoni, and G. Ciabattoni, *Measurement of 8-isoprostane in exhaled breath condensate.* Methods Mol Biol, 2010. **594**: p. 73-84.

73. Esterbauer, H., H. Schaur Rj Fau - Zollner, and H. Zollner, *Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes.* Free Radic Biol Med, 1991. **11**(1): p. 81-128.

74. Mittal, M., et al., *Reactive oxygen species in inflammation and tissue injury.* Antioxid Redox Signal, 2014. **20**(7): p. 1126-67.

75. Lee, J.W. and J.M. Davis, *Future applications of antioxidants in premature infants.* Curr Opin Pediatr, 2011. **23**(2): p. 161-6.

76. Brion, L.P., T.S. Bell Ef Fau - Raghuveer, and T.S. Raghuveer, *Vitamin E supplementation for prevention of morbidity and mortality in preterm infants.* Cochrane Database Syst Rev, 2003(4).

77. Pierce, M.R. and E. Bancalari, *The role of inflammation in the pathogenesis of bronchopulmonary dysplasia.* Pediatr Pulmonol, 1995. **19**(6): p. 371-8.

78. Vento, M., et al., *Preterm resuscitation with low oxygen causes less oxidative stress, inflammation, and chronic lung disease.* Pediatrics 2009. **124**(3): p. e439-49.

79. Saugstad, O.D., *Oxygen and oxidative stress in bronchopulmonary dysplasia.* J Perinat Med 2010. **38**(6): p. 571-7.

80. Inder, T.E., et al., *Lipid peroxidation as a measure of oxygen free radical damage in the very low birthweight infant.* Arch Dis Child Fetal Neonatal Ed, 1994. **70**(2): p. F107-11.

81. Tataranno, M.L., et al., *Resuscitating preterm infants with 100% oxygen is associated with higher oxidative stress than room air.* Acta Paediatr, 2015. **104**(8): p. 759-65.

82. Ahola, T., et al., *Plasma 8-isoprostane is increased in preterm infants who develop bronchopulmonary dysplasia or periventricular leukomalacia.* Pediatr Res, 2004. **56**(1): p. 88-93.

83. Boda, D., S. Nemeth I Fau - Pinter, and S. Pinter, *Surface tension, glutathione content and redox ratio of the tracheal aspirate fluid of premature infants with IRDS.* Biol Neonate, 1998. **74**(4): p. 281-8.

84. Dani, C., et al., *Superoxide dismutase and catalase activity in naturally derived commercial surfactants.* Pediatr Pulmonol, 2009. **44**(11): p. 1125-31.

85. Matalon, S., et al., *Characterization of antioxidant activities of pulmonary surfactant mixtures.* Biochim Biophys Acta

, 1990. **1035**(2): p. 121-7.

86. Madoglio, R.J., et al., *Inflammatory and oxidative stress airway markers in premature newborns of hypertensive mothers.* Braz J Med Biol Res, 2016. **49**(9): p. e5160.

87. Fabiano, A., et al., *The development of lung biochemical monitoring can play a key role in the early prediction of bronchopulmonary dysplasia.* Acta Paediatr, 2016. **105**(5): p. 535-41.

88. Collard, K.J., et al., *Pulmonary antioxidant concentrations and oxidative damage in ventilated premature babies.* Arch Dis Child Fetal Neonatal Ed, 2004. **89**(5): p. F412-6.

89. Rosso Mi Fau - Roark, S., et al., *Exhaled breath condensate in intubated neonates--a window into the lung's glutathione status.* Respir Res, 2014. **15**: p. 1.

90. Hitka, P., et al., *Assessment of exhaled gases in ventilated preterm infants.* Physiol Res, 2004. **53**(5): p. 561-4.

91. Mestan, K., et al., *Cord blood 8-isoprostane in the preterm infant.* Early Hum Dev, 2012. **88**(8): p. 683-9.

92. Negi, R., et al., *Evaluation of biomarkers of oxidative stress and antioxidant capacity in the cord blood of preterm low birth weight neonates.* J Matern Fetal Neonatal Med, 2012. **25**(8): p. 1338-41.

93. Mocatta, T.J., et al., *The effect of gestational age and labour on markers of lipid and protein oxidation in cord plasma.* Free Radic Res, 2004. **38**(2): p. 185-91.

94. Weinberger, B., et al., *Lipid peroxidation in cord blood and neonatal outcome.* Pediatr Int, 2006. **48**(5): p. 479-83.

95. Matthews, M.A., et al., *Increasing F2-isoprostanes in the first month after birth predicts poor respiratory and neurodevelopmental outcomes in very preterm infants.* J Perinatol, 2016. **36**(9): p. 779-83.

96. Cheah Fc Fau - Darlow, B.A., C.C. Darlow Ba Fau - Winterbourn, and C.C. Winterbourn, *Association of hydrogen peroxide in exhaled breath condensates from infants with respiratory distress syndrome with the development of chronic lung disease.* Arch Dis Child Fetal Neonatal Ed, 2006. **91**(2): p. F155.

97. Mourani, P.M. and S.H. Abman, *Pulmonary vascular disease in bronchopulmonary dysplasia: pulmonary hypertension and beyond.* Curr Opin Pediatr, 2013. **25**(3): p. 329-37.

98. Klinger, J.R., M.T. Abman Sh Fau - Gladwin, and M.T. Gladwin, *Nitric oxide deficiency and endothelial dysfunction in pulmonary arterial hypertension.* Am J Respir Crit Care Med, 2013. **188**(6): p. 639-46.

99. Meng, Q.H., et al., *Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis.* J Pathol, 1998. **184**(3): p. 323-31.

100. Grasemann, H., et al., *Decreased systemic bioavailability of L-arginine in patients with cystic fibrosis.* Respir Res, 2006. **7**(87).

101. Grasemann, H., et al., *L-ornithine derived polyamines in cystic fibrosis airways.* PLoS One, 2012. **7**(10): p. e46618.

102. Grasemann, H., et al., *Asymmetric dimethylarginine contributes to airway nitric oxide deficiency in patients with cystic fibrosis.* Am J Respir Crit Care Med, 2011. **183**(10): p. 1363-8.

103. Bode-Boger, S.M., L.J. Scalera F Fau - Ignarro, and L.J. Ignarro, *The L-arginine paradox: Importance of the L-arginine/asymmetrical dimethylarginine ratio.* Pharmacol Ther, 2007. **114**(3): p. 295-306.

104. Zakrzewicz, D. and O. Eickelberg, *From arginine methylation to ADMA: a novel mechanism with therapeutic potential in chronic lung diseases.* BMC Pulm Med, 2009. **9**: p. 5.

105. Carraro, S., et al., *Asymmetric dimethylarginine in exhaled breath condensate and serum of children with asthma.* Chest, 2013. **144**(2): p. 405-410.

106. Leiper, J., et al., *S-nitrosylation of dimethylarginine dimethylaminohydrolase regulates enzyme activity: further interactions between nitric oxide synthase and dimethylarginine dimethylaminohydrolase.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13527-32.

107. Kavurt, S., et al., *Increased ADMA levels are associated with poor pulmonary outcome in preterm neonates.* J Matern Fetal Neonatal Med, 2017. **30**(7): p. 864-869.

108. Trittmann, J.K., et al., *Plasma asymmetric dimethylarginine levels are increased in neonates with bronchopulmonary dysplasia-associated pulmonary hypertension.* J Pediatr, 2015. **166**(2): p. 230-3.

109. Richir, M.C., et al., *Plasma ADMA concentrations at birth and mechanical ventilation in preterm infants: a prospective pilot study.* Pediatr Pulmonol, 2008. **43**(12): p. 1161-6.

110. MacNee, W. and R.M. Tuder, *New paradigms in the pathogenesis of chronic obstructive pulmonary disease I.* Proc Am Thorac Soc, 2005. **2**(4): p. 258-66.

111. Schneibel, K.R., et al., *Inflammatory mediator patterns in tracheal aspirate and their association with bronchopulmonary dysplasia in very low birth weight neonates.* J Perinatol, 2013. **33**(5): p. 383-7.

112. Corradi, M., et al., *Aldehydes and glutathione in exhaled breath condensate of children with asthma exacerbation.* Am J Respir Crit Care Med, 2003. **167**(3): p. 395-9.

113. Horvath, I., et al., *Exhaled breath condensate: methodological recommendations and unresolved questions.* Eur Respir J, 2005. **26**(3): p. 523-48.

114. Horvath, I., et al., *A European Respiratory Society technical standard: exhaled biomarkers in lung disease. LID - 1600965 [pii] LID - 10.1183/13993003.00965-2016 [doi].* Eur Respir J, 2017. **49**(4).

115. van Mastrigt, E., J.C. de Jongste, and M.W. Pijnenburg, *The analysis of volatile organic compounds in exhaled breath and biomarkers in exhaled breath condensate in children - clinical tools or scientific toys?* Clin Exp Allergy, 2015. **45**(7): p. 1170-88.

116. Montuschi, P., et al., *NMR spectroscopy metabolomic profiling of exhaled breath condensate in patients with stable and unstable cystic fibrosis.* Thorax, 2012. **67**(3): p. 222-8.

117. Filippone, B.E., *Chronic lung disease after premature birth.* NEJM, 2007.

118. Kononikhin, A.S., et al., *Exhaled breath condensate analysis from intubated newborns by nano-HPLC coupled to high resolution MS.* J Chromatogr B Analyt Technol Biomed Life Sci, 2017. **1047**: p. 97-105.

119. Bertino, E., et al., *Neonatal anthropometric charts: the Italian neonatal study compared with other European studies.* J Pediatr Gastroenterol Nutr, 2010. **51**(3): p. 353-61.

120. Lee, H.C., J.B. Subeh M Fau - Gould, and J.B. Gould, *Low Apgar score and mortality in extremely preterm neonates born in the United States.* Acta Paediatr, 2010. **99**(12): p. 1785-9.

121. Malin, G.L., K.S. Morris Rk Fau - Khan, and K.S. Khan, *Strength of association between umbilical cord pH and perinatal and long term outcomes: systematic review and meta-analysis.* BMJ, 2010. **340**: p. c1471.

122. Jobe, A.H. and E. Bancalari, *Bronchopulmonary dysplasia.* Am J Respir Crit Care Med, 2001. **163**.

123. Folesani, G., et al., *Urea in exhaled breath condensate of uraemics and patients with chronic airway diseases.* Acta Biomed, 2008. **79**(1): p. 1:79-86.

124. Chen, Y., et al., *ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors.* Science, 2006. **314**(5806): p. 1792-5.

125. Okada, S.F., et al., *Physiological regulation of ATP release at the apical surface of human airway epithelia.* J Biol Chem, 2006. **281**(32): p. 22992-3002.

126. Esther, C.R., Jr., et al., *Elevated airway purines in COPD.* Chest, 2011. **140**(4): p. 954-960.

127. Esther, C.R., Jr., et al., *Mass spectrometric analysis of biomarkers and dilution markers in exhaled breath condensate reveals elevated purines in asthma and cystic fibrosis.* Am J Physiol Lung Cell Mol Physiol, 2009. **296**(6): p. L987-93.

128. Csoma, Z., et al., *Adenosine level in exhaled breath increases during exercise-induced bronchoconstriction.* Eur Respir J, 2005. **25**(5:): p. 873-8.

129. Esther, C.R., Jr., et al., *Extracellular purines are biomarkers of neutrophilic airway inflammation.* Eur Respir J, 2008. **31**(5): p. 949-56.

130. Yeh, M.Y., et al., *Chronic alcoholism alters systemic and pulmonary glutathione redox status.* Am J Respir Crit Care Med, 2007. **176**(3): p. 270-6.

131. Weinberger, B., et al., *Lipid peroxidation in cord blood and neonatal outcome.* (1328-8067 (Print)).

132. Perrone, S., et al., *Placental histological examination and the relationship with oxidative stress in preterm infants.* Placenta, 2016. **46**(1532-3102 (Electronic)): p. 72-78.

133. Casimirri, E., et al., *Biomarkers of oxidative-stress and inflammation in exhaled breath condensate from hospital cleaners.* Biomarkers, 2016. **21**(2): p. 115-22.

134. Sanli, C., et al., *Elevated homocysteine and asymmetric dimethyl arginine levels in pulmonary hypertension associated with congenital heart disease.* Pediatr Cardiol, 2012. **33**(8): p. 1323-31.

135. Hitka, P., et al., *Assessment of exhaled gases in ventilated preterm infants.* (0862-8408 (Print)).

136. Corradi, M. and A. Mutti, *Exhaled breath analysis: from occupational to respiratory medicine.* Acta Biomed, 2005. **76**(2): p. 20-9.

137. Vyas, A., et al., *The effect of temperature on exhaled breath condensate collection.* J Breath Res, 2012. **6**(3): p. 036002.

138. Andreoli, R., et al., *Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry.* (0951-4198 (Print)).

139. Effros, R.M., *Exhaled breath condensate: delusion or dilution?* Chest, 2010. **138**(3): p. 471-2.

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