Supplementary material

Title: Respiratory infection rates differ between geographically distant paediatric cystic fibrosis patient cohorts

Authors: Kathryn A. Ramsey[1,2*], Emily Hart[3,4*], Lidija Turkovic[1], Marc Padros-Goossens[1], Stephen M. Stick[1,5], Sarath C Ranganathan[2,4,6] on behalf of the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) [#]

AREST CF early surveillance protocol

All children diagnosed with CF in Western Australia are managed at Princess Margaret Hospital, Perth and children diagnosed in Victoria (apart from those in the southern metropolitan area) are managed at the Royal Children's Hospital, Melbourne. Over 95% of eligible children enrol into the AREST CF program. In Australia the diagnosis of CF is made in the majority of infants by six weeks of age, following detection by newborn screening [1]. The majority of infants have no respiratory symptoms at diagnosis but may have underlying pulmonary inflammation and infection [2].

The AREST CF early surveillance program includes assessment soon after diagnosis (approximately three months of age) and then annually until six years of age.

Assessments include:

- Lung function testing using infant lung function tests under chloral hydrate sedation at three months, one year and two years of age
- Chest CT scan at end inspiration (25 cmH_20) and end-expiration (0 cmH_20) under general anaesthetic
- Bronchoscopy and bronchoalveolar lavage (BAL) following the chest CT for assessment of pulmonary inflammation and infection

Parents are informed that this is a clinically-directed surveillance program with some research aspects. Parents are given the opportunity to consent to each aspect (clinical or research) of the program separately. The ethics committees of Princess Margaret Hospital, Perth and the Royal Children's Hospital, Melbourne have approved the program.

Bronchoscopy and bronchoalveolar lavage

A bronchoscopy was performed after the CT scan during which BAL fluid was collected. The right middle lobe was lavaged with three aliquots of saline (1 ml per kg body weight) and an additional aliquot lavaged into the lingula or the most affected lobe identified on CT [2, 3]. The first aliquot of BAL fluid was processed at each centre for the detection of bacteria, viruses and fungi using standard culture techniques. Subsequent aliquots were pooled for the quantification of pulmonary inflammation which included total and differential cell counts, and measurement of free NE activity (lower limit of detection = 200 ng/ml) [2].

Microbiology protocol

Using a sterile disposable pipette a drop (10 – 50ul) of BAL fluid was inoculated onto agar plates and spread for single colonies using a sterile loop. The plates were inoculated in the following manner: Blood agar, Cytosine Lactose Electrolyte Deficient (CLED) agar, and blood agar + ticarcillin (for resistant *P. aeruginosa* identification) were incubated at 35ºC for 48hrs in a CO2 incubator; PC plate (selective plate for *B.cepacia*) was incubated at 35ºC for 48hrs in a CO2 incubator, then at room temperature for another 24hrs; MSA (Mannitol salt agar for S. aureus) was incubated at 35ºC for 48hrs in an aerobic atmosphere; Sabarouds agar (for fungi isolation) was incubated at 35ºC for 48hrs in a CO2 incubator, then at 28ºC for 14 days; Fildes agar (for *Haemophilus* isolation) plates were incubated at 35ºC for 48hrs in an anaerobic environment (anaerobic incubation to prevent overgrowth by *P. aeruginosa*).

Gram stain was prepared and examined for bacteria, leucocytes and epithelial cells. Bacteria were identified by colony morphology, gram stain and biochemical tests including oxidase and C390 screening test specifically for *Pseudomonas. P. aeruginosa* were classified as smooth, rough or mucoid based on colony morphology on blood agar plates. *P. aeruginosa* colonies were unclassified if colony morphology was ambiguous. Growth was reported as isolated colonies, light, scanty, moderate or abundant growth based on the organism density on the plates which relate to colony counts of $<10^3$, 10^4, 10^5, 10^6, 10^7, respectively.

Sensitivity testing of all pathogens, including *P. aeruginosa* and, *S. aureus* were performed by the agar dilution breakpoint method according to Clinical Laboratory Standards Institute (CLSI) guidelines. Sensitivity of isolates such as *Haemophilus* was performed by disc testing using CSLI guidelines. A wet preparation was made from the BAL fluid and examined for fungal elements.

Immunofluorescence and culture methods were used to identify viral infections. For immunofluorescence, specimens were prepared by washing the cell pellet in PBS until a ‘tight’ cell pellet was obtained. A glass pipette was used to make a smear of the cells within four wells of an 8-well Teflon coated slide for RSV; Parainfluenzae 1,2&3; influenzae A&B; and adenovirus. The slide was air dried at room temperature and fixed in acetone. Direct Fluroescent Antibody Testing was carried out using commercially available monoclonal antibodies. If no evidence of viral infection was detected then BAL fluid was processed for viral cell culture. Virus was cultured by inoculation of an aliquot of BAL on to cultured mammalian cell lines grown on round glass coverslips. Virus was identified by staining the

cultured cells (on the coverslips) with monoclonal antibodies and examination of the cells under a fluorescence microscope.

Inflammation protocol

BAL fluid was pooled and centrifuged for 5 minutes at 1500 rpm. Aliquots of supernatant were stored at -80ºC until needed for further analysis. The cell pellet was washed if required and resuspended in 1ml PBS. Total cell count using a haemocytometer was performed on 10ul of cell suspension and viability assessed using equal volume of tryphan blue stain. Cytospins were performed on 10^6 cells/ml and stained using Leishman stain. Differential cell counts were performed on 300 consecutive cells at 100x magnification. The following cells were counted: macrophages, neutrophils, lymphocytes and eosinophils.

Samples frozen at -80ºC were transported to Perth from Melbourne overnight in an insulated container packed with dry ice to keep frozen. On arrival in Perth all samples were stored at -80ºC. Analysis of inflammatory mediators IL-1β, IL-12, IL-6, IL-8, IL-10 and TNFα were conducted using a standard cytometric bead array human inflammation kit (BD Biosciences, San Diego, CA) with a working range between 20 and 5000 pg/ml. Analysis of IL-8 was completed using an ELISA (BD Opt EIA, BD Biosciences, San Diego, CA) with a working range between 0.01 and 6.40 ng/ml. Free neutrophil elastase activity was assessed using an adapted ELISA. BAL fluid supernatant was serial diluted 1:2 with Tris buffer in duplicate. Tris buffer was the negative control and human neutrophil elastase diluted to 25 ug/ml was the standard. Substrate N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide (dissolved in NMP) was added to each well. Activity was read immediately at 450nm. The plate was then incubated at 37ºC in a CO_2 incubator and read again at 20, 30 and 40 minutes. The results were calculated using AssayZap and the best time point taken. The lower limit of detection for this assay was 200ηg/ml.

Computed tomography

Chest CT and BAL were performed under general anaesthesia. Children were intubated with a cuffed tracheal tube and a standardised recruitment manoeuvre, consisting of 10 consecutive slow breaths up to the total lung capacity (trans-respiratory pressure (P_{rs}) of 37 – 40 cmH_20) over a positive end-expiratory pressure of 5 cmH_20 for 1-2 seconds after each inspiration, was used to reduce procedure related atelectasis.

Following this, an inspiratory scan was obtained under a positive airway pressure of 25 cm H2O, after which an expiratory CT scan was obtained at end expiration (0 cm H2O). In Perth,

all inspiratory scans were obtained with a volumetric protocol, and expiratory scans were obtained with either a three-slice protocol (prior to 2010) or a volumetric protocol (from 2010). In Melbourne, inspiratory scans were obtained with either a volumetric protocol (prior to 2010) or a volumetric protocol (from 2010), and all expiratory scans were obtained with either a three-slice protocol. Details of the scanners and settings are have been described previously [5, 6].

All CT images were scored by an experienced paediatric thoracic radiologist at the Perth centre for the presence of structural lung disease using a simplified CF CT scoring method [2, 4]. Each scan was examined in six zones (upper, middle and lower areas of the left and right lungs). Each zone was scored for the presence and extent of bronchiectasis (outer-edge bronchus–artery cross-sectional area ratio >1), mucus plugging (high-density airway occlusion or tree-in-bud appearance), bronchial wall thickening (assessed subjectively as airway walls that are thicker than or have increased signal density relative to normal airways) visualized on inspiratory scans, and trapped air (geographic low-density regions) seen on expiratory scans.

Clinical data

A standardised respiratory symptoms questionnaire was conducted on the morning of the BAL in infants with CF to gain information on parentally reported symptoms (cough, upper respiratory tract infection, sputum production) and current medication use (including antibiotic use). A respiratory clinician also performed a physical examination of the infant and reported the presence or absence of wheeze, crackles or a respiratory tract infection. The number of days spent in hospital for a respiratory illness was determined using the clinical notes.

Infection control and treatment

Patients at both centres were seen at clinic every three months and had equivalent treatment strategies in terms of prophylactic antibiotics, chronic medication use, and airway clearance techniques (Supplemental Table 1). Perth and Melbourne CF centres followed the same infection control policy documented by the Australian Cystic Fibrosis Foundation. Both centres had a clinical policy to use anti-staphylococcal prophylaxis for a minimum of the first two years of life using amoxicillin/clavulanic acid to cover both S. *aureus* and H. *influenzae*; however, adherence to this policy was not determined objectively. During exacerbations treatment with amoxicillin/clavulanic acid was usually maintained for at least two weeks

before changed to a second line antibiotic if required. Second line antibiotics were prescribed when symptoms persisted for more than two weeks. In Perth second line antibiotic management was the addition of nebulised tobramycin to amoxicillin/clavulanic acid or nebulised tobramycin and oral ciprofloxacin with temporary discontinuation of amoxicillin/clavulanic acid for two to four weeks followed by intravenous tobramycin if necessary. In Melbourne, cefaclor was introduced as a second line antibiotic during exacerbations. In a minority (approximately 7%) this antibiotic was prescribed to replace amoxicillin/clavulanic acid at the outset of an exacerbation rather than later. Perth and Melbourne AREST CF centres had an early detection and eradication program for *P. aeruginosa*; once detected the child would receive two weeks of intravenous anti-pseudomonal antibiotics followed by four weeks of oral antibiotics and one to two months of inhaled tobramycin. A BAL sample was scheduled to determine eradication. In addition, in Perth, inhaled tobramycin for one month was prescribed for suspected infection with *P. aeruginosa* if an exacerbation had not responded to oral antibiotics.

Supplementary Table S1: Surveillance, infection control, and treatment practises between centres

	Melbourne	Perth
Visits	3 monthly clinic and annual surveillance visits	3 monthly clinic and annual surveillance visits
Infection control	Australian Cystic Fibrosis Foundation policy document	Australian Cystic Fibrosis Foundation policy document
Prophylactic antibiotics	Oral amoxicillin/clavulanic acid (0-2 years minumum)	Oral amoxicillin/clavulanic acid (0-2 years minumum)
Exacerbations	Oral amoxicillin/clavulanic acid (2 weeks) followed by nebulised tobramycin (2-4 weeks) Alternative: Nebulised tobramycin and oral ciprofloxacin (2-4 weeks) Followed by intravenous tobramycin if necessary	Oral amoxicillin/clavulanic acid (2 weeks) followed by oral cefaclor (2-4 weeks) Alternative: Oral cefaclor antibiotic (2-4 weeks) Followed by intravenous tobramycin if necessary
Eradication	Pseudomonas aeruginosa detected in BAL fluid: • Intravenous anti-pseudomonal antibiotics (2 weeks) • Followed by oral antibiotics (4 weeks) • Followed by inhaled tobramycin (1 - 2 months) • Follow up BAL to check for eradication	Pseudomonas aeruginosa detected in BAL fluid: • Intravenous anti-pseudomonal antibiotics (2 weeks) • Followed by oral antibiotics (4 weeks) • Followed by inhaled tobramycin (1 - 2 months) • Follow up BAL to check for eradication

References

1. Massie RJ, Curnow L, Glazner J, Armstrong DS, Francis I. Lessons learned from 20 years of newborn screening for cystic fibrosis. *Med J Aust* 2012: 196(1): 67-70.
2. Sly PD, Brennan S, Gangell C, de Klerk N, Murray C, Mott L, Stick SM, Robinson PJ, Robertson CF, Ranganathan SC. Lung disease at diagnosis in infants with cystic fibrosis detected by newborn screening. *Am J Respir Crit Care Med* 2009: 180(2): 146-152.
3. Gangell C, Gard S, Douglas T, Park J, de Klerk N, Keil T, Brennan S, Ranganathan S, Robins-Browne R, Sly PD. Inflammatory responses to individual microorganisms in the lungs of children with cystic fibrosis. *Clin Infect Dis* 2011: 53(5): 425-432.
4. Stick SM, Brennan S, Murray C, Douglas T, von Ungern-Sternberg BS, Garratt LW, Gangell CL, De Klerk N, Linnane B, Ranganathan S, Robinson P, Robertson C, Sly PD. Bronchiectasis in infants and preschool children diagnosed with cystic fibrosis after newborn screening. *J Pediatr* 2009: 155(5): 623-628 e621.
5. Mott LS, Park J, Murray CP, Gangell CL, de Klerk NH, Robinson PJ, Robertson CF, Ranganathan SC, Sly PD, Stick SM. Progression of early structural lung disease in young children with cystic fibrosis assessed using CT. *Thorax* 2012: 67(6): 509-516.
6. Rosenow T, Oudraad MC, Murray CP, Turkovic L, Kuo W, de Bruijne M, Ranganathan SC, Tiddens HA, Stick SM, Arest CF. PRAGMA-CF: a Quantitative Structural Lung Disease CT Outcome in Young Children with Cystic Fibrosis. *Am J Respir Crit Care Med* 2015: 191(10): 1158-1165.