NIH-PA Author Manuscript

Patterns of blood protein concentrations of ELGANs classified by three patterns of respiratory disease in the first two postnatal weeks

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Abstract

We examined the association between elevated concentrations of 25 blood proteins in blood spots collected on postnatal days 1, 7, and 14 from infants < 28 weeks gestation who survived to 24 months and the risk of two patterns of early lung disease i.e., early and persistent pulmonary dysfunction (EPPD), and normal early pulmonary function followed by pulmonary deterioration (PD). 38% (N=347) of our cohort had PD, and 43% (N=383) had EPPD. On postnatal day 14, elevated concentrations of two proteins (RANTES and VEGF) were associated with reduced risk of PD. Similarly, the risk of EPPD was also reduced if three proteins had elevated concentrations on postnatal day 14 (RANTES, MMP-1, and VEGF). In contrast, the risk of EPPD was increased if on day 14 two proteins had elevated concentrations (IL-8 and ICAM-1). Inflammation might influence the risk of EPPD and PD, or be a consequence of lung damage or therapies to minimize lung dysfunction.

Introduction

Early pulmonary dysfunction in extremely low gestational age newborns (<28 weeks gestation, ELGANs) can be characterized by three distinct patterns, based on the fraction of inspired oxygen they require in the first two postnatal weeks(1). A minority of ELGANs have relatively normal pulmonary function throughout the first two postnatal weeks (Low fraction of inspired oxygen, or FiO_2). Another group has pulmonary deterioration (PD), characterized by resolution of their initial lung disease during the first week, and deterioration during the second postnatal week, resulting in the need for increased supplemental oxygen. A third group has early and persistent pulmonary dysfunction (EPPD) requiring treatment with high concentrations of supplemental oxygen throughout the first two postnatal weeks.

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We previously investigated the clinical risk factors for each pattern of respiratory dysfunction. Low GA, fetal growth restriction, and receipt of mechanical ventilation on postnatal day 7 were associated with both PD and EPPD in the ELGAN Study sample (1). Confirmed bacteremia, receipt of vitamin A and surfactant, and treatment of patent ductus arteriosus (PDA) were associated with an increased risk of EPPD. However, histologic chorioamnionitis and funisitis were not associated with either PD or EPPD.

In our large cohort of ELGANs the risk of chronic lung disease (CLD) was lowest in children who had persistently low FiO_2, intermediate in those who developed PD, and highest in children who had EPPD (1). In this cohort, the risk of CLD is associated with elevated blood concentrations during the first two postnatal weeks of a variety of proteins integral to inflammation, including pro-inflammatory cytokines, adhesion molecules and matrix metalloproteinases (2). Others have also found that inflammation probably plays a role in the development of chronic lung disease (3, 4). These observations led us to hypothesize that systemic markers of inflammation, as manifested by blood concentrations of a variety of inflammatory proteins, would be elevated in the EPPD group and elevated to a lesser degree in the PD group compared to infants in the Low FiO_2 group.

Methods

The ELGAN Study

The infants included in this analysis are a subset of infants enrolled in a multi-center epidemiologic study to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in Extremely Low Gestational Age Newborns (the ELGAN Study) (1, 5, 6). From March 2002 to August 2004, women delivering before the 28th week of gestation at one of 14 participating institutions were asked to enroll in the study. Individual institutional review boards (including The University of North Carolina at Chapel Hill; see acknowledgements for full list of institutions) approved the enrollment and consent processes. The original consent and approval included the provision for secondary analyses of de-identified data. Therefore, separate approval was not necessary for the analyses reported in this manuscript. The subset for this study included all infants enrolled in the ELGAN Study for whom sufficient information was available to determine an early respiratory group assignment and measurements of proteins in a blood specimen were obtained during the first two postnatal weeks. We limited our sample of newborns to the 908 who survived to 24 months because protein measurements were available only for children who had a developmental assessment at 24 months post-term equivalent. A full description of the methods is provided elsewhere (1, 5–7). Here we focus on those most relevant to the current study.

Newborn variables

The GA estimates were based on a hierarchy of the quality of available information. Most desirable were estimates based on the dates of embryo retrieval or intrauterine insemination or fetal ultrasound before the 14th week (62%). When these were not available, reliance was placed sequentially on a fetal ultrasound at 14 or more weeks (29%), last menstrual period without fetal ultrasound (7%), and GA recorded in the log of the neonatal intensive care unit (1%).

ELGANs were classified into three mutually exclusive groups: those with consistently low FiO_2 (a FiO_2 consistently < 0.23 on <u>all</u> days between 3 and 7 postnatal days and receiving $FiO_2 \leq 0.25$ on Day 14), those with PD (a $FiO_2 < 0.23$ on <u>any</u> days between 3 and 7 days and receiving $FiO_2 > 0.25$ on day 14), and those with EPPD (a FiO_2 consistently ≥ 0.23 on

all days between 3 and 7 postnatal days and receiving $FiO_2 > 0.25$ on Day 14) (1, 6). For individual infants, the FiO_2 assigned for each day of life was the mode FiO_2 for that day.

Blood spot collection

Drops of blood were collected on filter paper (Schleicher & Schuell 903, Keene, NH) after specimens were obtained for clinical indications between postnatal days 1–3, 5–8, and 12–15. Dried blood spots were stored at −70°C in sealed bags with desiccant until processed.

Elution of proteins from blood spots

For protein elution, 12mm punched biopsies of the frozen blood spots were submerged in 300 µL phosphate buffered saline containing 0.1% Triton X100 (Sigma-Aldrich, St. Louis, MO) and 0.03% Tween-20 (Fisher, Hampton, NH), vortexed for 30 seconds, and incubated on a shaker for 1 hour at 4°C. The buffer and biopsy were then transferred over the filter of a SpinX tube (Corning Fisher), centrifuged at $2000 \times g$ followed by collection of the filtered eluted blood. An additional wash of the punch was performed in 100 µL for a final elution volume of 400 µL.

Protein measurements

The ELGAN Study was designed to identify antecedents of brain injury in ELGANs. Thus all the proteins to be measured in the blood spots were chosen because of their potential to provide information about the risks and consequences of brain damage, and because they could be measured with validity and reasonable precision. Blood spot collection, elution of proteins from blood spots, and the electrochemiluminescence protein-measurement procedures are described in detail elsewhere (8, 9). The electrochemiluminescence system provides measurements comparable to those obtained with traditional ELISA(10, 11). In addition, split quality control blood pools tested on each plate showed inter-assay variation of < 20% for each protein.

We measured CRP (C-Reactive Protein), SAA (serum amyloid A), MPO (myeloperoxidase), IL-1β; IL-6; IL-6R; TNF-α; TNF-R1; TNF-R2; IL-8 (CXCL8); MCP-1 (monocyte chemoattractant protein-1; CCL2), MCP-4 (monocyte chemoattractant protein-4; CCL13), MIP-1B (macrophage inflammatory protein-1 β; CCL4), RANTES (regulated upon activation, normal T-cell expressed, and secreted; CCL5), I-TAC (interferon-inducible T cell α-chemoattractant; CXCL11), ICAM-1 (CD54), ICAM-3 (CD50), VCAM-1 (vascular cell adhesion molecule-1; CD106), E-SEL (E-selectin; CD62E), MMP-1 (matrix metalloproteinase-1), MMP-9 (matrix metalloproteinase-9), VEGF, VEGF-R1, VEGF-R2, and IGFBP-1 (insulin growth factor binding protein-1).

Data analysis

We evaluated the generalized null hypothesis that the occurrence of PD and EPPD are not associated with blood protein concentrations in the highest quartile for GA on the day the blood was collected. We began our analyses by exploring the frequency of each entity in the 4 quartiles of the concentration of each protein. Rarely was a linear trend seen. Thus, the analyses we present here compare children whose protein concentration was in the highest quartile to children whose protein concentration was in the lower 3 quartiles.

The risk of each pattern of respiratory disease entity associated with a protein concentration in the highest quartile is presented as an OR and the 99% confidence interval. We selected this confidence interval because we wanted to modify our analyses for multiple comparisons (25 proteins measured at 3 times) thereby avoiding a type I (false positive) error, while not appreciably increasing the risk of a type II (false negative) error (12). Because PD and

EPPD are mutually exclusive, and each is appropriately compared to the same referent group (children in the low FiO_2 group), we used multinomial logistic regression (13).

Because protein concentrations can be influenced by processes associated with earlier events, we created logistic regression models in which protein concentrations are temporally ordered, so that the earliest occurring protein concentrations are entered first and are not displaced by later protein concentrations (14–19). For these time-oriented risk models (TORMs), we categorize sets of protein concentrations by the day the blood spot was obtained. Each set is called an epoch and are labeled 1, 7 and 14, corresponding to the days on which blood spots were collected. Endogenous variables, GA at birth, and birth weight Z-score, were entered in epoch 1. We used a step down procedure seeking a parsimonious solution without interaction terms.

Results

Of the 1506 infants enrolled in the ELGAN Study, 1302 infants had information available to determine the respiratory pattern in the first two postnatal weeks (see Figure 1). Of these, 908 survived to 24 months, had proteins measured on one or more days, and were included in the analyses presented here. Among these infants, 19% (N=172) were in the Low FiO_2 group, 38% (N=347) were in the PD group, and 42% (N=383) were in the EPPD group. Five percent of children in the Low FiO_2 group died before 24 months, compared to 9% in the PD group and 16% in the EPPD group. CLD developed in 16% of children in the Low FiO_2, 51% of children in the PD group, and 70% of children in the EPPD group.

Multinomial analyses of risk of PD versus Low FiO_2 (Table 1)

Elevated concentrations of none of the 25 inflammation-related proteins that were measured were associated with increased risk of PD. A decreased risk of PD was found if the newborn had elevated day-7 concentrations of RANTES (CCL5), VCAM-1 (CD106) or MMP-1, or elevated day-14 concentrations of RANTES (CCL5) or VEGF.

Multinomial analyses of risk of EPPD versus Low FiO_2 (Table 1)

A reduced risk of EPPD was found among infants with elevated day-1 concentrations of RANTES (CCL5), elevated day-7 concentrations of MIP-1β (CCL4) or VEGF, or elevated day-14 concentrations of IL-8 (CXCL8) or ICAM-1 (CD54). An increased risk of EPPD was found among infants with elevated day-7 concentrations of MCP-1 (CCL2) and those with elevated day-14 concentrations of IL-8 (CXCL8) or ICAM-1 (CD54). RANTES was the only protein whose concentration appeared to be inversely related to EPPD risk on all days.

Time-oriented regression (Table 2)

In time-oriented regression, PD was not associated with any day-1 protein elevations, but a reduced risk of PD was associated with an elevation of VCAM-1 in day-7 blood as well as with an elevation of VEGF in day-14 blood. In the EPPD versus Low FiO_2 time-oriented risk model (Table 2), elevated concentrations of RANTES on day 1 and day 7, and of SAA on day 7 were associated with a decreased risk of EPPD. However, an elevated concentration of ICAM-1 on day 7 was associated with increased risk.

Discussion

We examined the association between elevated concentrations of 25 blood proteins in the first two postnatal weeks and the risk of EPPD and PD among extremely preterm infants. In light of evidence that pulmonary inflammation contributes to the occurrence of CLD (3, 20)

we hypothesized that systemic inflammation would be present in infants who developed EPPD, and to lesser degree in infants who developed PD.

Only a small amount of predictive information about PD and EPPD is available in blood spots collected on day 1, with increasing information on day 7, and even more on day 14. The number of inflammation-related proteins associated with EPPD increased with increasing postnatal age raising the possibility that systemic inflammation associated with lung dysfunction reflects postnatal phenomena. This is no surprise since infants who experience EPPD have such inflammation-promoting exposures as supplemental oxygen and mechanical ventilation, which are viewed as antecedents of CLD (21, 22).

Elevations of inflammation-associated proteins provided more information about reduced risk of PD rather than increased risk. No protein concentration elevation on any of the three days was associated with increased risk of PD. In contrast, the elevated concentrations of three proteins on day 7 (RANTES, VCAM-1 and MMP-1) and two on day 14 (RANTES and VEGF) were associated with reduced risk. On one level, this is evidence that inflammation does not contribute substantially to the occurrence of PD. Our findings raise the possibility that inflammation protects. We are reluctant to accept this view just yet. Rather, we think our provocative findings need to be replicated and alternative explanations need to be considered.

RANTES has been described as pleiotropic (23). Some of the functions of this protein contribute to reducing inflammation (24, 25). Consequently, our finding elevated concentrations of RANTES associated with reduced risk of EPPD and PD might be viewed as additional support for a modulation of pre-existing inflammation not otherwise apparent to us. Low concentrations of RANTES in blood shortly after birth have also been associated with increased risk of BPD or death (3, 4). Elevated concentrations of VEGF were associated with reduced risk of PD. Support for the hypothesis that low VEGF levels contribute to PD comes from the observation that VEGF preserves alveolar development, and that reduced concentrations of lung VEGF disrupt lung growth and vascular distributions within the lung (26, 27).

As with PD, elevations of some inflammation-associated proteins, including RANTES and VEGF, are associated with a decreased risk of EPPD. However, in contrast to PD, elevations of some proteins are associated with an increased risk of EPPD. On day 14, elevated concentrations of two proteins (IL-8 and ICAM-1) were associated with increased risk of EPPD. The association with IL-8 is not surprising because of its pro-inflammatory properties. ICAM-1 is an adhesion molecule that mediates the adhesion of white blood cells to the vascular endothelium and is important for recruiting leukocytes to sites of inflammation. Higher concentrations of soluble ICAM-1 have been associated with poor outcomes in adults with acute lung injury (28).

Our findings are best interpreted in light of the current understanding of the biology of inflammation. Individual proteins are only a small part of a much larger process. Inflammation-related proteins are highly interrelated(29), and together appear to contribute to organ damage (30). As a result, each protein is best interpreted as representing the interlinked web of proteins indicating inflammation, and possibly promoting lung damage or phenomena likely to reduce damage. An inflammatory stimulus alters the expression of more than a thousand genes, increasing the expression of hundreds of proteins, and decreasing the expression of hundreds of others(31). For example, when the expression of VCAM-1 is increased, so, too, is the expression of other markers of inflammation (32). Consequently, what we see is a very limited snapshot of what has preceded and/or accompanied the development of lung dysfunction.

Lung dysfunction can promote and/or exacerbate an inflammatory response. Hypercapnia promotes adhesion molecule expression in both inflammatory-stimulated human pulmonary microvascular endothelial cells and in an animal model of inflammatory-induced acute lung injury (33). This, and similar observations, suggest that some of the protein concentration changes we found might be a consequence, rather than an antecedent of lung dysfunction. Such a response might very well further contribute to lung dysfunction, as lung inflammation can promote more lung inflammation. Lung exposed to an inflammatory stimulus releases circulating mediators that induce liver vascular endothelial cells to activate NF-κB and to express ICAM-1 and VCAM-1(34). In essence, a self-reinforcing loop is generated that promotes sustained inflammation, thereby increasing the risk of inflammation-induced damage.

We do not know to what extent protein concentrations in the blood are of pulmonary origin. It is possible that under certain circumstances proteins produced by cells in the lung remain in the lung compartment, while under other circumstances (e.g., intense or prolonged pulmonary inflammation), they enter the circulation. It is possible that infants with PD and EPPD have inflammatory processes that are more prominent in the lung than in the blood. That is, systemic inflammation is not a prominent component of early pulmonary dysfunction and our examination of proteins in the blood may provide only a limited and biased view of the inflammatory processes associated with early pulmonary dysfunction. Analysis of inflammatory proteins from the epithelial lung fluid might help elucidate this possibility.

Our study has several strengths and limitations. We created time-oriented risk models to provide information about the relative significance of protein concentrations at critical postnatal ages. We included a large number of infants, making it unlikely that we have missed important associations due to lack of statistical power. We selected infants based on GA, not birth weight, in order to minimize confounding due to factors related to fetal growth restriction (35).

The weaknesses of our study are those of all observational studies. We do not know to what extent the protein reductions or elevations contributed to PD or EPPD and to what extent the protein reductions or elevations are surrogates for other processes that might have influenced CLD risk. We did not include infants who died prior to 24 months, and more infants died in the EPPD group and PD group than in the Low FiO_2 group. This might bias our results if infants died because of a systemic inflammatory process. In addition, we collected all of our data prospectively. Eluting materiel from blood spots is likely to include the intracellular contents of disrupted cells in the circulation. Thus, what we measured is probably the sum of proteins in the circulation, and proteins released from cells whose integrity was lost in the drying of the blood spots.

In conclusion, elevated concentrations of inflammation-associated proteins in the blood were associated with reduced risk of PD. A similar pattern was seen to a lesser extent among children who had EPPD, although elevated concentrations of other inflammation-related proteins were associated with increased risk. The lack of evidence of inflammation in the blood early in the neonatal period among children who had EPPD or PD suggests that systemic inflammation is not a process that begins *in utero*. Postnatal events or exposures are more likely to be the promoters of this phenomenon. Because of the strong association between EPPD and CLD, and to a lesser degree PD and CLD, targeted therapies designed to modulate specific elements of systemic inflammation may modify CLD risk.

Acknowledgments

Abbreviations

CLD	chronic lung disease
ELGAN	extremely low gestational age newborn
EPPD	early and persistent pulmonary dysfunction
FiO2	fraction of inspired oxygen
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
PD	pulmonary deterioration
SAA	serum amyloid A
VCAM	vascular cell adhesion molecule

REFERENCES

1. Laughon M, Allred EN, Bose C, O'Shea TM, Van Marter LJ, Ehrenkranz RA, Leviton A. Patterns of respiratory disease during the first 2 postnatal weeks in extremely premature infants. Pediatrics. 2009; 123:1124–1131. [PubMed: 19336371]

2. Bose C, Laughon M, Allred EN, Van Marter LJ, O'Shea TM, Ehrenkranz RA, Fichorova R, Leviton A. for the ELGAN Study Investigators. Blood protein concentrations in the first two postnatal weeks that predict bronchopulmonary dysplasia among infants born before the 28th week of gestation. Pediatr Res. 2011; 69:347–353. [PubMed: 21150694]

3. Bose CL, Dammann CE, Laughon MM. Bronchopulmonary dysplasia and inflammatory biomarkers in the premature neonate. Arch Dis Child Fetal Neonatal Ed. 2008; 93:F455–F461. [PubMed: 18676410]

4. Ambalavanan N, Carlo WA, D'Angio CT, McDonald SA, Das A, Schendel D, Thorsen P, Higgins RD. Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. Pediatrics. 2009; 123:1132–1141. [PubMed: 19336372]

5. Laughon M, Bose C, Allred E, O'Shea TM, Van Marter LJ, Bednarek F, Leviton A. Factors associated with treatment for hypotension in extremely low gestational age newborns during the first postnatal week. Pediatrics. 2007; 119:273–280. [PubMed: 17272616]

6. Laughon M, Bose C, Allred EN, O'Shea TM, Ehrenkranz RA, Van Marter LJ, Leviton A. Antecedents of chronic lung disease following three patterns of early respiratory disease in preterm infants. Arch Dis Child Fetal Neonatal Ed. 2011; 96:F114–F120. [PubMed: 20688867]

7. O'Shea TM, Allred EN, Dammann O, Hirtz D, Kuban KC, Paneth N, Leviton A. The ELGAN study of the brain and related disorders in extremely low gestational age newborns. Early Hum Dev. 2009; 85:719–725. [PubMed: 19765918]

8. Leviton A, Fichorova R, Yamamoto Y, Allred EN, Dammann O, Hecht J, Kuban K, McElrath T, O'Shea TM, Paneth N. Inflammation-related proteins in the blood of extremely low gestational age newborns. The contribution of inflammation to the appearance of developmental regulation. Cytokine. 2011; 53:66–73. [PubMed: 20934883]

9. Hecht JL, Fichorova RN, Tang VF, Allred EN, McElrath TF, Leviton A. Relationship between neonatal blood protein concentrations and placenta histologic characteristics in extremely low GA newborns. Pediatr Res. 2011; 69:68–73. [PubMed: 20921924]

10. Fichorova RN, Richardson-Harman N, Alfano M, Belec L, Carbonneil C, Chen S, Cosentino L, Curtis K, Dezzutti CS, Donoval B, Doncel GF, Donaghay M, Grivel JC, Guzman E, Hayes M, Herold B, Hillier S, Lackman-Smith C, Landay A, Margolis L, Mayer KH, Pasicznyk JM, Pallansch-Cokonis M, Poli G, Reichelderfer P, Roberts P, Rodriguez I, Saidi H, Sassi RR, Shattock R, Cummins JE Jr. Biological and technical variables affecting immunoassay recovery of cytokines from human serum and simulated vaginal fluid: a multicenter study. Anal Chem. 2008; 80:4741–4751. [PubMed: 18484740]

11. Fichorova RN, Trifonova RT, Gilbert RO, Costello CE, Hayes GR, Lucas JJ, Singh BN. Trichomonas vaginalis lipophosphoglycan triggers a selective upregulation of cytokines by human female reproductive tract epithelial cells. Infect Immun. 2006; 74:5773–5779. [PubMed: 16988255]

12. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology. 1990; 1:43–46. [PubMed: 2081237]

13. Dubin N, Pasternack BS. Risk assessment for case-control subgroups by polychotomous logistic regression. Am J Epidemiol. 1986; 123:1101–1117. [PubMed: 3706280]

14. Leviton A, Pagano M, Kuban KC, Krishnamoorthy KS, Sullivan KF, Allred EN. The epidemiology of germinal matrix hemorrhage during the first half-day of life. Dev Med Child Neurol. 1991; 33:138–145. [PubMed: 2015981]

15. Leviton A, Kuban KC, Pagano M, Allred EN, Van Marter L. Antenatal corticosteroids appear to reduce the risk of postnatal germinal matrix hemorrhage in intubated low birth weight newborns. Pediatrics. 1993; 91:1083–1088. [PubMed: 8502506]

16. Leviton A, Paneth N, Reuss ML, Susser M, Allred EN, Dammann O, Kuban K, Van Marter LJ, Pagano M. Hypothyroxinemia of prematurity and the risk of cerebral white matter damage. J Pediatr. 1999; 134:706–711. [PubMed: 10356138]

17. Leviton A, Dammann O, Allred EN, Kuban K, Pagano M, Van Marter L, Paneth N, Reuss ML, Susser M. Antenatal corticosteroids and cranial ultrasonographic abnormalities. Am J Obstet Gynecol. 1999; 181:1007–1017. [PubMed: 10521769]

18. Leviton A, Paneth N, Reuss ML, Susser M, Allred EN, Dammann O, Kuban K, Van Marter LJ, Pagano M, Hegyi T, Hiatt M, Sanocka U, Shahrivar F, Abiri M, DiSalvo D, Doubilet P, Kairam R, Kazam E, Kirpekar M, Rosenfeld D, Schonfeld S, Share J, Collins M, Genest D, Heller D, Shen-Schwarz S. Maternal infection, fetal inflammatory response, and brain damage in very low birthweight infants. Pediatr Res. 1999; 46:566–575. [PubMed: 10541320]

19. Laptook AR, O'Shea TM, Shankaran S, Bhaskar B. Adverse neurodevelopmental outcomes among extremely low birth weight infants with a normal head ultrasound: prevalence and antecedents. Pediatrics. 2005; 115:673–680. [PubMed: 15741371]

20. Speer CP. Chorioamnionitis, postnatal factors and proinflammatory response in the pathogenetic sequence of bronchopulmonary dysplasia. Neonatology. 2009; 95:353–361. [PubMed: 19494557]

21. Hillman NH, Moss TJ, Kallapur SG, Bachurski C, Pillow JJ, Polglase GR, Nitsos I, Kramer BW, Jobe AH. Brief, large tidal volume ventilation initiates lung injury and a systemic response in fetal sheep. Am J Respir Crit Care Med. 2007; 176:575–581. [PubMed: 17641159]

22. Johnson BH, Yi M, Masood A, Belcastro R, Li J, Shek S, Kantores C, Jankov RP, Tanswell AK. A critical role for the IL-1 receptor in lung injury induced in neonatal rats by 60% O2. Pediatr Res. 2009; 66:260–265. [PubMed: 19542903]

23. Levy JA. The unexpected pleiotropic activities of RANTES. J Immunol. 2009; 182:3945–3946. [PubMed: 19299688]

24. Gatt ME, Urieli-Shoval S, Preciado-Patt L, Fridkin M, Calco S, Azar Y, Matzner Y. Effect of serum amyloid A on selected in vitro functions of isolated human neutrophils. J Lab Clin Med. 1998; 132:414–420. [PubMed: 9823935]

25. Shahrara S, Park CC, Temkin V, Jarvis JW, Volin MV, Pope RM. RANTES modulates TLR4-induced cytokine secretion in human peripheral blood monocytes. J Immunol. 2006; 177:5077–5087. [PubMed: 17015691]

26. Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in

human infants dying with bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001; 164:1971–1980. [PubMed: 11734454]

27. Thébaud B, Ladha F, Michelakis ED, Sawicka M, Thurston G, Eaton F, Hashimoto K, Harry G, Haromy A, Korbutt G, Archer SL. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. Circulation. 2005; 112:2477–2486. [PubMed: 16230500]

28. Calfee CS, Eisner MD, Parsons PE, Thompson BT, Conner ER Jr, Matthay MA, Ware LB. Soluble intercellular adhesion molecule-1 and clinical outcomes in patients with acute lung injury. Intensive Care Med. 2009; 35:248–257. [PubMed: 18670758]

29. Sriskandan S, Altmann DM. The immunology of sepsis. J Pathol. 2008; 214:211–223. [PubMed: 18161754]

30. Wang H, Ma S. The cytokine storm and factors determining the sequence and severity of organ dysfunction in multiple organ dysfunction syndrome. Am J Emerg Med. 2008; 26:711–715. [PubMed: 18606328]

31. Zak DE, Aderem A. Systems biology of innate immunity. Immunol Rev. 2009; 227:264–282. [PubMed: 19120490]

32. Haverslag R, Pasterkamp G, Hoefer IE. Targeting adhesion molecules in cardiovascular disorders. Cardiovasc Hematol Disord Drug Targets. 2008; 8:252–260. [PubMed: 19075635]

33. Liu Y, Chacko BK, Ricksecker A, Shingarev R, Andrews E, Patel RP, Lang JD Jr. Modulatory effects of hypercapnia on in vitro and in vivo pulmonary endothelial-neutrophil adhesive responses during inflammation. Cytokine. 2008; 44:108–117. [PubMed: 18713668]

34. Markovic N, McCaig LA, Stephen J, Mizuguchi S, Veldhuizen RA, Lewis JF, Cepinskas G. Mediators released from LPS-challenged lungs induce inflammatory responses in liver vascular endothelial cells and neutrophilic leukocytes. Am J Physiol Gastrointest Liver Physiol. 2009; 297:G1066–G1076. [PubMed: 19815624]

35. Arnold CC, Kramer MS, Hobbs CA, McLean FH, Usher RH. Very low birth weight: a problematic cohort for epidemiologic studies of very small or immature neonates. Am J Epidemiol. 1991; 134:604–613. [PubMed: 1951265]
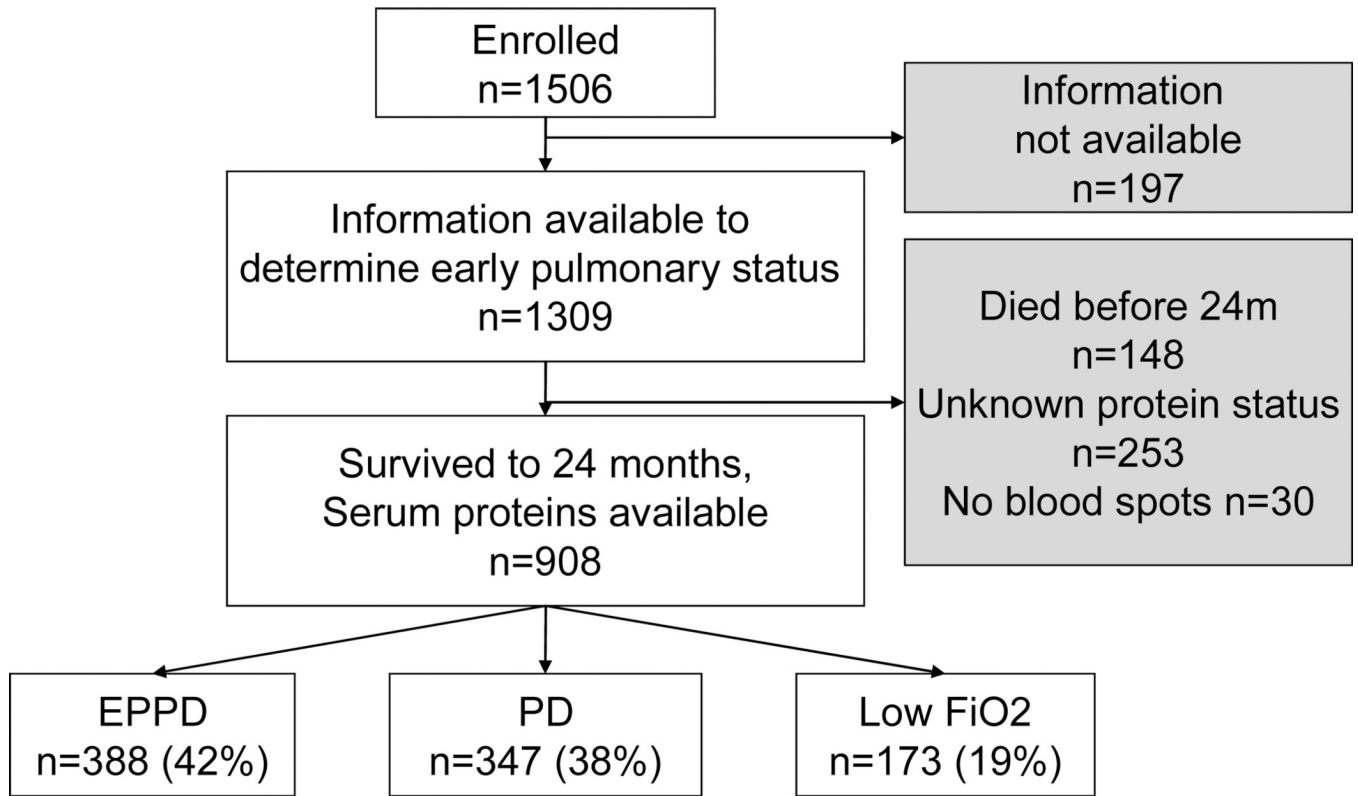
Figure 1.
Derivation of cohort

Table 1

ORs (99% confidence intervals) of PD and EPPD associated with protein concentrations in the top quartile (in groups of children classified by GA and day blood was obtained). These are the result of multinomial analyses comparing children who developed PD, and those who had EPPD to those who had persistently low FiO$_2$ and are adjusted for GA (23–24, 25–26, 27 weeks).

	PD (pulmonary deterioration)			EPPD (early and persistent pulmonary dysfunction)		
	Day 1	**Day 7**	**Day 14**	**Day 1**	**Day 7**	**Day 14**
CRP	1.3 (0.7, 2.4)	1.2 (0.7, 2.2)	0.9 (0.5, 1.7)	0.8 (0.5, 1.6)	1.5 (0.8, 2.7)	1.1 (0.6, 2.1)
SAA	1.3 (0.7, 2.4)	0.7 (0.4, 1.3)	0.8 (0.4, 1.4)	1.0 (0.6, 1.9)	0.6 (0.3, 1.03)	0.8 (0.4, 1.5)
MPO	1.0 (0.6, 1.7)	1.2 (0.7, 2.1)	0.8 (0.4, 1.4)	0.9 (0.5, 1.6)	1.0 (0.6, 1.8)	1.1 (0.6, 1.9)
IL-1β	1.1 (0.6, 1.9)	0.9 (0.6, 1.6)	1.1 (0.6, 2.1)	1.1 (0.6, 1.9)	0.9 (0.5, 1.6)	1.3 (0.7, 2.4)
IL-6	1.0 (0.6, 1.8)	1.0 (0.6, 1.8)	0.7 (0.4, 1.2)	0.9 (0.5, 1.5)	1.1 (0.6, 2.0)	1.0 (0.6, 1.9)
IL-6R	1.0 (0.5, 1.7)	0.7 (0.4, 1.2)	0.6 (0.4, 1.2)	1.3 (0.7, 2.3)	1.0 (0.6, 1.7)	0.7 (0.4, 1.4)
TNF-α	1.0 (0.6, 1.7)	1.0 (0.5, 1.7)	1.0 (0.6, 1.9)	1.1 (0.6, 1.9)	1.2 (0.7, 2.1)	1.6 (0.8, 2.9)
TNF-R1	0.8 (0.5, 1.5)	0.8 (0.5, 1.5)	1.0 (0.5, 2.0)	1.0 (0.6, 1.9)	1.1 (0.6, 1.9)	1.3 (0.7, 2.5)
TNF-R2	1.1 (0.6, 2.1)	0.9 (0.5, 1.6)	0.8 (0.5, 1.6)	1.4 (0.8, 2.5)	1.1 (0.6, 2.1)	0.9 (0.5, 1.7)
Il-8 (CXCL8)	1.3 (0.7, 2.3)	1.1 (0.6, 2.1)	1.1 (0.5, 2.1)	1.1 (0.6, 2.1)	1.4 (0.8, 2.6)	**2.1 (1.1, 4.0)**
MCP-1 (CCL2)	0.9 (0.5, 1.6)	1.4 (0.8, 2.6)	1.2 (0.6, 2.2)	0.9 (0.5, 1.6)	**2.2 (1.2, 4.2)**	1.8 (0.9, 3.4)
MCP-4 (CCL13)	0.7 (0.4, 1.3)	0.8 (0.4, 1.4)	0.6 (0.3, 1.04)	1.0 (0.6, 1.8)	1.1 (0.6, 2.0)	0.6 (0.3, 1.1)
MIP-1β (CCL4)	0.9 (0.5, 1.5)	0.7 (0.4, 1.2)	0.6 (0.4, 1.1)	0.9 (0.5, 1.5)	*0.5 (0.3, 0.9)*	0.6 (0.3, 1.1)
RANTES (CCL5)	0.6 (0.4, 1.1)	*0.6 (0.3, 0.99)*	*0.5 (0.3, 0.9)*	*0.4 (0.3, 0.8)*	*0.4 (0.2, 0.7)*	*0.3 (0.2, 0.6)*
I-TAC (CXCL11)	0.6 (0.4, 1.1)	0.8 (0.5, 1.4)	0.6 (0.3, 1.1)	0.7 (0.4, 1.2)	0.8 (0.4, 1.4)	0.6, (0.3, 1.1)
ICAM-1 (CD54)	0.9 (0.5, 1.7)	1.3 (0.7, 2.4)	1.6 (0.8, 3.4)	1.0 (0.6, 1.8)	1.7 (0.9, 3.1)	**3.4 (1.6, 6.9)**
ICAM-3 (CD50)	0.9 (0.5, 1.6)	0.9 (0.5, 1.6)	0.8 (0.4, 1.4)	0.9 (0.5, 1.7)	0.8 (0.4, 1.4)	0.9 (0.5, 1.6)
VCAM-1 (CD106)	0.8 (0.4, 1.4)	*0.5 (0.3, 0.95)*	0.7 (0.4, 1.2)	1.1 (0.6, 1.9)	0.7 (0.4, 1.3)	0.9 (0.5, 1.6)
E-SEL (CL62E)	0.7 (0.4, 1.3)	1.3 (0.7, 2.4)	1.0 (0.6, 1.9)	0.9 (0.5, 1.6)	1.7 (0.9, 3.0)	1.1 (0.6, 2.1)
MMP-1	0.8 (0.4, 1.3)	*0.5 (0.3, 0.9)*	0.6 (0.3, 1.1)	0.7 (0.4, 1.3)	0.6 (0.4, 1.1)	*0.5 (0.3, 0.97)*
MMP-9	0.9 (0.5, 1.6)	1.0 (0.6, 1.8)	1.0 (0.5, 2.0)	1.1 (0.6, 1.9)	0.7 (0.4, 1.3)	1.4 (0.8, 2.7)
VEGF	0.9 (0.5, 1.6)	0.6 (0.4, 1.1)	*0.5 (0.3, 0.9)*	1.0 (0.6, 1.8)	*0.6 (0.3, 0.97)*	*0.4 (0.2, 0.8)*
VEGF-R1	0.7 (0.4, 1.2)	0.9 (0.5, 1.5)	0.7 (0.4, 1.3)	1.0 (0.6, 1.8)	1.0 (0.6, 1.8)	0.7 (0.4, 1.2)
VEGF-R2	0.8 (0.4, 1.3)	0.8 (0.5, 1.4)	0.8 (0.5, 1.5)	0.8 (0.4, 1.4)	0.7 (0.4, 1.2)	0.8 (0.4, 1.4)

	PD (pulmonary deterioration)			EPPD (early and persistent pulmonary dysfunction)		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
IGFBP-1	0.9 (0.5, 1.6)	1.2 (0.7, 2.3)	0.8 (0.4, 1.4)	1.0 (0.6, 1.9)	1.4 (0.7, 2.5)	0.9 (0.5, 1.6)

Table 2

ORs (and 99% confidence intervals) of pulmonary deterioration (PD) and early and persistent pulmonary dysfunction (EPPD) associated with a concentration of the protein on the left in the highest quartile for GA and day blood was obtained relative to the risk among children whose concentration was in the lower 3 quartiles. PD and EPPD were separately compared to Low FiO_2. These are time-oriented logistic risk models which included, before model reduction, all proteins listed in Table 1, GA (23–24, 25–26, 27 weeks), and birth weight Z-score (BWZ) < −1 (entered with the day 1 proteins).

Pulmonary deterioration (PD)				
		ORs (99% confidence intervals)		
Day	**Protein**	**Day 1**	**Days 1 and 7**	**Day 1, 7, 14**
1	GA 23–24	**6.6 (2.2, 20)**	**6.8 (2.3, 20)**	**9.0 (2.5, 32)**
	GA 25–26	**1.9 (1.1, 3.3)**	**2.0 (1.2, 3.4)**	**2.0 (1.1, 3.7)**
	BWZ < -1	1.3 (0.6, 2.7)	1.3 (0.6, 2.7)	1.5 (0.6, 3.6)
7	VCAM-1		*0.5 (0.3, 0.99)*	0.7 (0.3, 1.2)
14	VEGF			*0.5 (0.3, 0.9)*
Early and persistent pulmonary dysfunction (EPPD)				
		ORs (99% confidence intervals)		
Day	Protein	Day 1	Days 1 and 7	Days 1, 7, 14
1	GA 23–24	**25 (8.4, 76)**	**38 (10, 143)**	**69 (13, 370)**
	GA 25–26	**4.2 (2.3, 7.4)**	**4.4 (2.3, 8.2)**	**5.2 (2.5, 11)**
	BWZ < -1	2.0 (0.9, 4.1)	2.2 (0.9, 5.0)	2.1 (0.9, 4.9)
	RANTES	0.4 (0.2, 0.8)	*0.5 (0.3, 1.03)*	0.5 (0.2, 1.2)
7	RANTES		*0.4 (0.2, 0.9)*	0.5 (0.2, 1.2)
	SAA-2		*0.4 (0.2, 0.8)*	*0.4 (0.2, 1.00)*
	ICAM-1		**3.0 (1.4, 6.5)**	2.0 (0.8, 5.1)
14	ICAM-1			**2.9 (1.2, 7.2)**
	RANTES			*0.3 (0.1, 0.6)*