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BIOMARKERS OF RESPIRATORY SYNCYTIAL VIRUS (RSV) INFECTION: SPECIFIC NEUROTROPHIN AND CYTOKINE LEVELS PROVIDE INCREASED ACCURACY IN PREDICTING DISEASE SEVERITY

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SUMMARY

Despite fundamental advances in the research on respiratory syncytial virus (RSV) since its initial identification almost 60 years ago, recurring failures in developing vaccines and pharmacologic strategies effective in controlling the infection have allowed RSV to become a leading cause of global infant morbidity and mortality. Indeed, the burden of this infection on families and health care organizations worldwide continues to escalate and its financial costs are growing. Furthermore, strong epidemiologic evidence indicates that early-life lower respiratory tract infections caused by RSV lead to the development of recurrent wheezing and childhood asthma. While some progress has been made in the identification of reliable biomarkers for RSV bronchiolitis, a "one size fits all" biomarker capable of accurately and consistently predicting disease severity and post-acute outcomes has yet to be discovered. Therefore, it is of great importance on a global scale to identify useful biomarkers for this infection that will allow pediatricians to cost-effectively predict the clinical course of the disease, as well as monitor the efficacy of new therapeutic strategies.

Keywords

Asthma; Brain-derived growth factor (BDNF); Bronchiolitis; Lung development; Nerve growth factor (NGF)

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections (LRTI) in infants and pre-school children. As virtually all children are infected with this virus at least once by 2 years of age, current estimates suggest RSV infection is responsible for greater than 60% of all acute LRTI in young children and more than 80% of all acute LRTI in infants under one year old worldwide [1, 2]. RSV LRTI are typically hallmarked by the development of bronchiolitis, defined as inflammation of the bronchiolar airways leading to obstruction [3]. Accordingly, bronchiolitis is the principal cause of hospitalization

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in infants under one year of age in the U.S., accounting for 16.4% of total hospitalizations in this age group [1], while more than one third of children will have developed bronchiolitis at some point prior to becoming two years old [4]. The clinical severity of bronchiolitis ranges from mild forms manageable on an outpatient basis to severe cases that require mechanical ventilation or extracorporeal membrane oxygenation (ECMO) in intensive care units (ICU) [2]. Highlighting the potential threat posed by bronchiolitis, a recent retrospective study found the ICU admission rate for all children under age 2 with bronchiolitis to be 22%, with 89% of the ICU admission cases testing positive for RSV infection and 47% ultimately requiring mechanical ventilation [5].

Early-life RSV infection has also been shown to lead to chronic airway dysfunction, with an increased risk of subsequent wheezing episodes that lasts for several years after the acute infection [6-9]. This characteristic of the virus has been the basis of a longstanding debate as to whether RSV infection is simply a trigger that uncovers an intrinsic predisposition, or rather a causative agent contributing to the inception of childhood asthma [10, 11]. In addition to a plethora of studies focusing on the role of innate and adaptive immune system, other studies have looked into the effects of RSV on airway innervation and reactivity both during and after infection and have shown that specific neurotrophic proteins and their cognate receptors are upregulated by this infection leading to persistent airway hyperreactivity [12-14]. In addition, the establishment of a lytic RSV infection cycle has been shown to involve a series of pro-survival mechanisms - including host immune system evasion and prevention of apoptosis by infected host cells – mediated through modulation of nerve growth factor (NGF) activity [12, 15-17].

Even with intensive research focused on RSV since its discovery in 1956 [18, 19], pediatric clinicians currently lack an effective vaccine capable of preventing the infection and must instead rely on supportive therapies aimed at managing complications of bronchiolitis (e.g., nasal suctioning, hydration, supplemental oxygen) [2, 20]. Once the infection is established, clinicians will empirically assess the trajectory of a patient’s symptoms in an attempt to predict its evolution, and will tailor their management strategy accordingly [21]. Over time, clinical observation has led to the identification of specific risk factors for severe RSV bronchiolitis: premature birth (<32 weeks gestational age), male sex, lack of breastfeeding, chronic lung disease, hemodynamically-significant congenital heart disease, and severe immunodeficiency amongst others [22-28]. However, numerous studies have also demonstrated that a majority of children hospitalized with RSV bronchiolitis lack these risk factors and were healthy prior to their clinical event [5, 29, 30]. As a result, efforts aimed at identifying novel biological markers measurable in the host during the course of RSV bronchiolitis have multiplied in hopes of developing a clinically effective method to predict disease severity outcomes. To this end, some advances in the development of biomarkers of RSV bronchiolitis have been made over the last several years and the primary purpose of this review is to state the need and discuss potential solutions for such biomarkers in more detail.

CHARACTERISTICS OF RSV INFECTION

The highly contagious nature of RSV stems from its ability to survive outside of a host up to 6 hours on hard surfaces and on the skin for 20 minutes [31]. This prolonged viability permits the inoculation of mucous membranes in the eyes or nose and allows the virus to spread quickly from the hands of adult hosts to infants and young children [32, 33]. Upon inoculation, RSV first infects the nasopharyngeal epithelium of the upper respiratory tract and subsequently spreads towards its more competent replication site in the bronchiolar epithelium within 1-3 days with peak infectivity achieved around 5-days post-inoculation [15, 34]. Host innate and adaptive immune responses are continuously triggered during this spread to the lower airways, but fail to effectively clear RSV in young children and immunocompromised individuals [23, 35, 36].

The inherent ability of RSV to manipulate host innate, adaptive, and memory immune responses is a contributing factor to an inability to develop a fully effective vaccine against the virus [17]. From initial failings in vaccine development leading to increased mortality in 1966 [37, 38] to more recent RSV vaccine approaches that initially showed promise [20], there still remains a void of safe and effective prophylaxis, other than the passive protection provided by the humanized monoclonal antibody palivizumab (Synagis®; marketed by MedImmune, LLC) [39-43]. As the prohibitive costs of this intervention limit its use to a minority of high-risk infants, the need for research aimed at identifying reliable biomarkers of RSV bronchiolitis in parallel with safe and effective therapy remain strong [44].

IMPACT OF RSV INFECTION ON PEDIATRIC HEALTH

In the U.S. population under one year of age, RSV-associated LRTI account for approximately 126,000 hospitalizations (25.2 per 1,000) [45] with estimated direct costs ranging from $394 million to $1.1 billion annually [46, 47]. When examining the global burden of RSV infection on developing nations, a systematic review and analysis of available literature estimated that nearly 33.8 million new cases of RSV-associated LRTI occur worldwide in children under 5 years of age leading to approximately 3.4 million hospitalizations annually [48]. These figures also fail to take into account the rates of hospitalization and mortality that RSV infection imparts on the elderly, a population now recognized as extremely susceptible to the virus and for which the associated health care costs are surely tremendous [49-53]. When taken together on a global scale, high RSV-associated LRTI hospitalization rates coupled with the increasing price of healthcare potentially cost the world hundreds of billions of dollars annually and put significant strain on infected individuals, health care providers, and health care systems alike.

In the U.S., mortality associated with RSV infection is uncommon in the 21st century due to effective hygiene and supportive care, with the approximately 40 deaths per year mostly occurring in infants with complex chronic conditions or in those with life-threatening conditions preexisting the infection [54]. In contrast, the global impact of RSV infection on infants and young children is staggering: worldwide, RSV-associated LRTI are the second leading cause of pathogenic mortality in infants less than one year of age [48]. Varied estimates place the global mortality rate somewhere between 200,000 and 1 million deaths

annually in children under 5 years of age [2, 48]. Therefore, the need for effective strategies aimed at reducing RSV bronchiolitis morbidity and mortality rates has never been greater with the development and implementation of these tactics holding the potential to save hundreds of thousands of lives around the globe each year.

RSV INFECTION AND ASTHMA DEVELOPMENT

In immunocompetent children, RSV is fully cleared from the lower airways within 8 days after initial infection though its ability to cause persistent airway dysfunction is well documented [31, 55]. Multiple prospective epidemiological studies have associated these respiratory sequelae, chiefly recurrent episodes of wheezing and asthma development within the first decade of life, with early-life RSV LRTI [9-11, 56]. Mechanistic studies have demonstrated the involvement of both neurological networks [14, 57-60] and immune cells [61-63] within infected host tissues as being directly involved in post-infection airway obstruction. Numerous studies have also underscored the important effects that NGF and other neurotrophins have on airway tissues such as smooth muscle and fibroblasts during both acute bronchiolitis and chronic asthma [64-66]. The interplay between neural and immune networks has been described in numerous publications [2, 55, 61] and has led to continued and vigorous debate within the field of pediatric asthma as to which avenue has a greater impact on chronic sequelae development: nerves and nerve-derived proteins or immune effector cells and the cytokines associated with their responses.

NEUROTROPHINS AS BIOMARKERS OF RSV INFECTION

Neurotrophins are a family of proteins responsible for neuronal differentiation and survival that are continuously present in all vertebrates from the early embryonic stage forward throughout life [67, 68]. The neurotrophins family includes four highly conserved proteins with similar structure and function: NGF, brain-derived neurotrophic factor (BDNF), NT3, and NT4. While all of the classical NTs can bind to the low-affinity $p75^{NTR}$ receptor, each also preferentially binds specific high-affinity receptors of the tropomyosin-related tyrosine kinase (Trk) family, being TrkA (NGF), TrkB (BDNF and NT4) and TrkC (NT3) [69-72]. All of the classical NTs and their cognate receptors are widely expressed within numerous structural, neuronal, and auxiliary cells of the lungs [73, 74] where they have been found to contribute to a number of lung pathologies (e.g. bronchopulmonary dysplasia and asthma) either with their direct effect on neuronal structure and function or by interacting with multiple components of the immune system [15, 73, 75-83].

Elevated expression of NGF within the lower airways has been shown in animal models of early-life RSV infection **(Table 1)** [14, 57, 84]. Other studies focused on BDNF expression during neonatal lung pathologies associated with childhood asthma development also found high levels of BDNF and/or its cognate receptor TrkB [85, 86]. In a more recent animal study on vertical transmission during pregnancy, RSV was shown to cross the placental barrier where it could directly infect the lung buds of developing fetuses leading to increased airway expression of NGF and acetylcholine that persists through development and predisposes to airway hyperreactivity upon early-life RSV re-challenge [12]. The increased airway reactivity following vertical RSV transmission was found to be critically dependent

on the NGF-TrkA axis, as blockade of either using anti-NGF antibodies or the Trk-dependent signal transduction inhibitor K252a returned post-natal response to methacholine challenge to normal range.

The relevance of this animal data for human disease was confirmed by a clinical study of mechanically ventilated infants who underwent bronchoalveolar lavage (BAL), concluding that patients with acute RSV infections had significantly elevated levels of NGF and BDNF proteins in the cellular fraction of their BAL compared to mechanically ventilated non-infected infants and infants with respiratory failure caused by adenoviral or parainfluenza infections **(Figure 1)** [13]. At 48 hours post-intubation, NGF in the RSV-positive LRTI group was 7-fold higher compared with the RSV-negative LRTI group ($p < 0.001$) and 5-fold higher compared with the group without LRTI ($p < 0.001$). At the same time points, absolute BDNF concentrations were one order of magnitude larger than NGF concentrations, and were 12-fold higher in the RSV-positive LRTI group compared with the RSV-negative LRTI group ($p < 0.05$) and 5-fold higher compared with the group without LRTI ($p < 0.01$).

Cytologic analysis of the BAL showed no significant differences in absolute and differential leukocyte counts between RSV-positive and RSV-negative infants with LRTI, suggesting that the increased synthesis of neurotrophic factors derives from RSV-infected structural airway cells rather than bone marrow-derived inflammatory cells recruited from the circulation to fight the infection. However, strong TrkA immunofluorescence was detected both in desquamated epithelial cells and macrophages recovered from RSV-infected airways, whereas the NGF receptor was virtually undetectable in cells recovered from RSV-negative infants, either non-infected or infected with non RSV viruses. Of importance, these preparations were examined by a pathologist blind to the illness groups, who detected immunofluorescence staining in all specimens from RSV-positive patients, whereas all specimens from RSV-negative patients were all read as negative. Collectively, these data suggest that NGF-TrkA axis expression levels in the lower airways could be used as a sensitive and specific biomarker of acute RSV infection. Although it cannot be ruled out whether other pathogens have similar neurotrophic effects, the finding that RSV-negative LRTI was not associated with changes in neurotrophic pathways suggests that this effect may be unique to RSV, or at least shared by specific pathogens only.

In contrast, no change was detected in serum samples and BAL supernatants collected during the first 48 hours after intubation in the same patients, suggesting that these specimens reflect only indirectly the actual concentrations of neurotrophic factors present in the cells and tissues where they are produced, secreted, and involved in biological functions. However, in a subgroup of RSV-infected infants requiring mechanical ventilation for more than 48 hours, significantly higher NGF concentrations were measured in serum samples obtained at 72 hours **(Figure 2)**, suggesting that the transfer of locally secreted neurotrophins to the systemic circulation is gradual, and becomes measurable only later in the course of the infection and/or only in infants with more prolonged and severe RSV LRTI. This interpretation would also explain why higher NGF levels were found in the blood [87] and BAL fluid [88] of patients with chronic asthma, as in our study serum neurotrophin levels may have been low because the sampling was done early in the course of the illness.

Therefore, although blood samples are much more accessible than tracheal or bronchoalveolar lavage fluid, systemic concentrations of neurotrophic protein levels tend to increase less and later in the course of RSV disease compared to local airway expression, making their sensitivity and usefulness more problematic. Elevated levels of NGF were also found in human airways after allergen challenge [89] reinforcing the idea that this protein plays an important role in the pathophysiology of airway inflammation and hyperreactivity. Furthermore, strong airway expression of neurotrophic factors and receptors has been shown in active sarcoidosis [90] and in lung cancer [91], suggesting that neurotrophins not only are essential for neuro-immune integration, but are also controlling growth and differentiation of lung cells.

A relatively recent and surprising twist in this story has revealed that the lungs may not be the only source of RSV-induced NGF **(Table 2)**. Indeed, akin to animal studies displaying RSV infection penetrating extrapulmonary sites [12], a wealth of clinical evidence in humans exists which supports the notion that this infection is not limited to its primary target in the lungs. To date, RSV RNA has been detected in peripheral blood mononuclear cells (PBMCs), cerebrospinal fluid (CSF), myocardium, primary nerve cells, the middle ear, the liver, and bone marrow [92-100]. Once seeded in these extrapulmonary sites RSV infection can cause a number of important secondary pathologies, such as sino-atrial blockade [101] or increased liver enzymes and hepatitis development [99], though the exact mechanisms behind these occurrences are not known. Similar to RSV, the recent finding of latent *Mycobacterium tuberculosis* infection within human bone marrow stem cells [102] supports the idea that the bone marrow may serve as a sanctuary for latent RSV infection from which persistent post-bronchiolitis airway dysfunction, failures in development of immunity to the virus, and possibly even later reactivation arise [103].

Taken together, these studies have demonstrated that RSV can invade multiple extrapulmonary targets where one of its primary survival mechanisms within infected host cells would involve increased expression of the anti-apoptotic protein NGF. The specific pattern of RSV-induced NGF overexpression in infected tissues warrants an expansion of research into the potential of NGF as a useful biomarker to assess RSV bronchiolitis severity in humans. Going one step further, if NGF overexpression during RSV infection promotes virus survival in the short-term while also leading to long-term development of post-bronchiolitis airway dysfunction, then drug design strategies successfully inhibiting this pathway in humans could reshape our approaches towards treating RSV bronchiolitis and airway dysfunction.

CYTOKINES AS BIOMARKERS OF RSV INFECTION

There is no question that host immune response and subsequent lower respiratory tract inflammation during the progression of RSV infection play a key role in determining the severity of disease witnessed in patients [37, 38]. There is a continuous presence of lymphocytes and phagocytes both resident in the airway tissues and circulating within the peripheral bronchial and pulmonary vasculature, which serve as sentries to respond swiftly to any pathogenic challenge reaching the lower airways [104]. During RSV infection, these immune sentinels respond through cytokine signaling to activate the downstream cascade

that leads to infiltration of the bronchiolar airways by neutrophils, eosinophils macrophages, B-lymphocytes, $CD4^+$ and $CD8^+$ T cells [104-106]. Both animal and human studies of RSV infection have suggested that a T helper 2 (Th2)-polarized immune response may be generated in certain conditions, leading to the activation of cytotoxic $CD8^+$ T cells [62, 63] as well as the synthesis of interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13) [107-111].

While $CD8^+$ T cell presence is detectable in the lower airways during RSV infection, the functional cytotoxicity of these cells may be compromised due to the infiltration of neutrophils and eosinophils resulting from local Th2 cytokine expression [112, 113] as well as by the virus itself which is inherently capable of blocking the activation, proliferation, and function of $CD8^+$ T cells [104, 114-119]. The result of this multifactorial inhibition of $CD8^+$ T cells, accompanied by parallel reductions in overall Th1 immune response functionality, is a substantial reduction in viral clearance and increased severity of disease. The host immune response to RSV and the accompanying inflammation that is produced within the lower airways due to immune effector cells infiltration are much more important that the direct cytopathic effects of the virus in determining the symptoms of RSV disease. As a corollary, RSV infection in immunocompromised hosts usually presents with lower levels of wheeze and higher probability for invasive pneumonia, whereas immunocompetent hosts tend to display greater levels of airway obstruction and wheezing but no progression to the pulmonary parenchyma [94, 120].

In immunocompetent subjects, the host immune system is ultimately able to clear the virus from lung tissues and mitigate the course of disease severity. However, the immunologic memory developed during an acute infection with RSV is incomplete and does not prevent reinfection of the host even within the same epidemic season, though it does appear to reduce the overall severity of recurrent RSV infections [121-124]. What level of protection to RSV infection is conferred correlates well with the serum level of neutralizing antibodies, a finding confirmed by multiple clinical studies of human infants [125-128] and further reinforced by the finding that decreased serum anti-RSV immunoglobulin titers are associated with increased incidence of severe disease [123]. These findings explain why prophylaxis with the humanized monoclonal antibody palivizumab (Synagis®) is a safe and effective tool for clinicians to protect those early childhood populations carrying the most significant risk of developing severe RSV bronchiolitis [20, 42, 43].

Human blood plasma and serum are clinical sample sources possessing numerous qualities that make them of significant importance to clinicians. They are easily attainable in most situations and circumstances and across age groups, procurement methods are of generally low cost, they are well-preserved long term, many commercial kits are available for testing of a plethora of proteins, and the data gathered from these tests represent systemic levels which are more indicative of overall body-wide affects. Therefore, clinical blood plasma and serum testing are powerful methods for biomarker monitoring of RSV bronchiolitis in an effort to predict disease severity outcomes, and several host cytokines display differential blood plasma and serum levels during RSV infection **(Table 3)**. Levels of interleukin-8 (IL-8), interferon-gamma (IFN-γ) and the chemokine (C-C motif) ligand 5 (CCL-5) have been shown to be predictors of mechanical ventilator requirement during RSV infection and

bronchiolitis [129, 130]. Low levels of plasma TNF-α and IL-6 were correlated with increased infant hospitalization [131, 132], with peripheral blood mononuclear cell proliferation also being associated with increased infant ICU admission during RSV bronchiolitis [133] and $CD4^+$ T cell count predictive of mechanical ventilator requirement [129]. While the use of tracheal aspirate fluid (TAF) as a diagnostic tool during RSV bronchiolitis course is only possible in specific clinical situations, it has been shown to harbor potential biomarkers of severity with one study finding increased IL-6 and IL-17 levels in the TAF of infants with severe RSV bronchiolitis [134].

LEUKOTRIENES AS BIOMARKERS OF RSV INFECTION

The acute inflammatory response of airways infected by RSV in early life involves the release of cysteinyl leukotrienes and activation of the cysLT1 receptor, as manifested by the anti-inflammatory effect of the receptor antagonist montelukast in animal models [135] and in human studies [136]. Following the early phase of the viral respiratory infection, leukotriene production and release rapidly return to baseline levels, but can be reactivated when airborne irritants (e.g., tobacco smoke) stimulate nociceptive nerve fibers connected to the numerous mast cells still present in the lung tissues. Indeed, LTC_4 is elevated in the nasopharyngeal secretions of children during acute RSV infection and correlates with clinical severity, with more appreciable LTC_4 elevation in children displaying higher degrees of lower respiratory tract involvement [137, 138]. Accordingly, the terminal urine metabolite of cysteinyl leukotrienes (LTE_4) is elevated in the urine of young infants with RSV bronchiolitis as compared to controls without respiratory infection [139], and a study in intubated patients with severe RSV bronchiolitis found that increased LTE_4 in endotracheal aspirates correlates well with urinary LTE_4 levels [140]. An important practical implication of these findings is that the increased excretion of LTE_4 during acute bronchiolitis could not only be used to assess severity, but also to assist in the decision of starting therapy with leukotriene modifiers that have been shown to have therapeutic value in this setting, and maybe monitor evolution of the disease and response to therapy **(Table 4)**.

LTE_4 is the end-product of the 5-LO pathway in activated mast cells, eosinophils, and monocytes [141]. LTA_4, the primary 5-LO metabolite, is converted to LTC_4 and sequentially to LTD_4 and LTE_4 in the host cell. This metabolism is rapid and complete, in that LTC_4 is virtually undetectable in plasma. Because of its short half-life (approximately 7 minutes), LTE_4 is likewise difficult to detect in plasma as a consequence of the low rate of production and rapid elimination [142]. Normal human urine contains low but detectable amounts of LTE_4, ranging from 10 to 60 pg/ml [143]. Asthmatic patients with an acute episode of bronchoconstriction may have elevations of urinary LTE_4 to several hundred pg/ml, although their baseline concentration is not consistently abnormal. LTE_4 titration in urinary samples also avoids risks of *ex vivo* formation of metabolites during or after sampling, which may be a major problem when measuring arachidonic acid metabolism in other biological specimens.

The overproduction of cysteinyl leukotrienes reflected by urinary LTE_4 is more prominent in the younger patients infected with RSV [139]. Also, an intrinsic predisposition to develop

atopy or airway hyperreactivity seems to amplify the effect of the virus, as higher LTE_4 concentrations were found in infants with a medical history of eczema or dry cough and/or family history of asthma. In particular, eczema and dry cough had the strongest association with urinary LTE_4 levels in multivariate analysis. Age or atopy per se did not affect leukotriene synthesis in the absence of infection. Another important advantage of LTE_4 monitoring in the setting of RSV infections is that this biomarker is not affected by extrinsic environmental factors, such as pre- and/or post-natal exposure to tobacco smoke [139] or environmental pollution [144], as such factors do not affect leukotriene synthesis, either alone or in combination with the viral infection. New prospective, controlled studies are needed to establish whether urinary LTE_4 is more sensitive or specific than historical data alone in identifying young infants who will develop recurrent wheezing or asthma after RSV bronchiolitis, or in identifying potential responders to anti-leukotriene therapy for this infection.

ADDITIONAL BIOMARKERS SHOWING PROMISE

The lack of any single biomarker of RSV bronchiolitis that displays adequate predictive value and can be applied to all clinical scenarios is a recurring problem and has led many clinicians and researchers to pursue alternative strategies **(Table 4)**. A predictive model designed to identify which bronchiolitis patients within an emergency department are most likely to be admitted used concentration measurements of lactate dehydrogenase in nasal washings, which was found to have a positive predictive value of 88% [145]. Increased liver transaminase and alanine aminotransferase have been observed in children with RSV bronchiolitis [146], while decreased blood sodium levels accompanied by elevated antidiuretic hormone (ADH) levels have been described in patients specifically having RSV LRTI compared to RSV infections of the upper respiratory tract [147-149].

In addition, RSV titers have shown some limited promise as predictors of bronchiolitis severity, whether hailing from the blood [150, 151] or from nasal swabs [152-156]. More specific to active RSV infection, viral RNA has been detected in human PBMCs from infants and children with RSV LRTI [93-95], and RSV RNA levels within PBMCs were correlated with disease severity in a murine model [94]. More recently, the advent of next-generation whole genome sequencing coupled with the decreased cost and increased ease of performing these powerful assays has driven some investigators to conclude that identifying unique genetic traits specific to each patient affected by RSV bronchiolitis is as equally important as targeting the virus itself. This has led to one study of specific host gene expression patterns within the nasopharyngeal region during RSV infection progression [157], whereas others have sought to assess RSV disease severity in infants by generating whole blood gene expression profiles [158].

In the latter multi-center observational study, researchers analyzed whole blood RNA sequencing profiles from children under 2 years of age positively identified through clinical testing as being infected with either RSV, human rhinovirus (hRV), or influenza A. Their model distinguished infants with RSV LRTI versus LRTI from hRV or influenza A with 95% accuracy. More importantly as this study relates to RSV, patients with RSV LRTI displayed unique transcriptional profiles compared to healthy controls hallmarked by

significant overexpression of gene modules related to inflammation and innate immunity (including monocyte, neutrophil, and innate immune response genes) and decreased expression of gene modules related to adaptive immunity (including B cell and cytotoxic T/natural killer cell genes).

During the acute phase, patients with RSV LRTI displayed the highest levels of neutrophil-related gene expression paralleled by significant suppression of B cell, T cell, and lymphoid lineage response gene expression levels, which were either strikingly milder or even absent in patients with hRV or influenza A LRTI. Sub-classifying RSV LRTI by disease severity and tracking out to 1-month post-hospitalization, it was found that most host immune response gene patterns, such as those related to T cell and lymphoid lineage responsiveness, had ablated whereas the levels of B cell gene expression remained persistently suppressed during follow-up visits. This last finding provides further evidence as to the ability of RSV to modulate host immune responses with effects that specifically target anti-RSV immunoglobulin producing B cells and persist well after the period of symptomology and viral shedding/infectivity has subsided.

FUTURE RESEARCH DIRECTIONS

- Continue to research the full scope of NGF activity during acute RSV bronchiolitis, as well as during the post-bronchiolitis phase in which persistent airway dysfunction occurs.

- Continue to delineate the pathways and processes by which extrapulmonary sites become directly infected with RSV, what roles virus-mediated NGF expression may play in these sites, and what the immediate and long-term consequences to the health of the host are as a result of extrapulmonary RSV infections.

- Continued research using next generation whole genome sequencing to profile and pattern host immune responses at the gene level during RSV bronchiolitis will most likely yield significant advances in our understanding of how the virus modulates the ability of our immune system during the acute phase of infection and possibly for the weeks, months, and years that follow.

- Coupling this trend in host immune response gene research with studies of bone marrow and other extrapulmonary site RSV infections may yet elucidate the exact mechanism(s) behind the long-term suppression of host immune B-cell and memory responses to the virus.

- By understanding how host immunity to RSV is being evaded, we may be able to identify novel therapeutic targets for intervention both prior to and during RSV infection, which could ultimately lead to the development of new anti-viral pharmacologic strategies as well as a safe and effective vaccine.

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EDUCATIONAL AIMS

The reader will come to:

- Understand that RSV infection of the lower airways is the primary cause of acute bronchiolitis in infants and children worldwide.

- Learn the evidence linking early-life RSV lower respiratory tract infections to the development of childhood asthma.

- Recognize the multifaceted role of nerve growth factor (NGF) and other neurotrophins in the pathophysiology of RSV bronchiolitis.

- Know that a limited number of host biomarkers have been identified recently as predictive of RSV bronchiolitis severity outcomes.

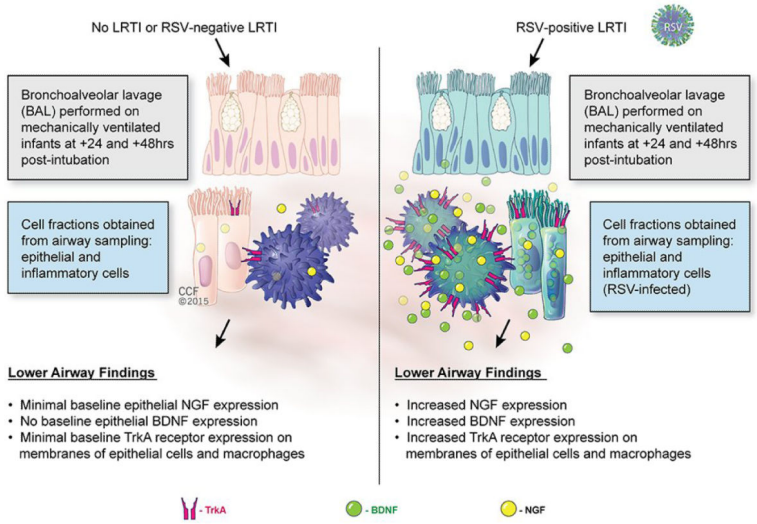

Figure 1. Neurotrophins as biomarkers of RSV bronchiolitis

Patients with acute RSV bronchiolitis have significantly higher concentrations of NGF and BDNF proteins in the cellular fractions sampled from their airways compared to non-infected infants or infants with acute bronchiolitis caused by adenoviral or parainfluenza infections whose airways express minimal baseline levels of both neurotrophins. Furthermore, epithelial cells and macrophages from the airways of RSV-infected infants have strong surface expression of the high-affinity NGF receptor TrkA, whereas in the absence of RSV infection this receptor is virtually absent.

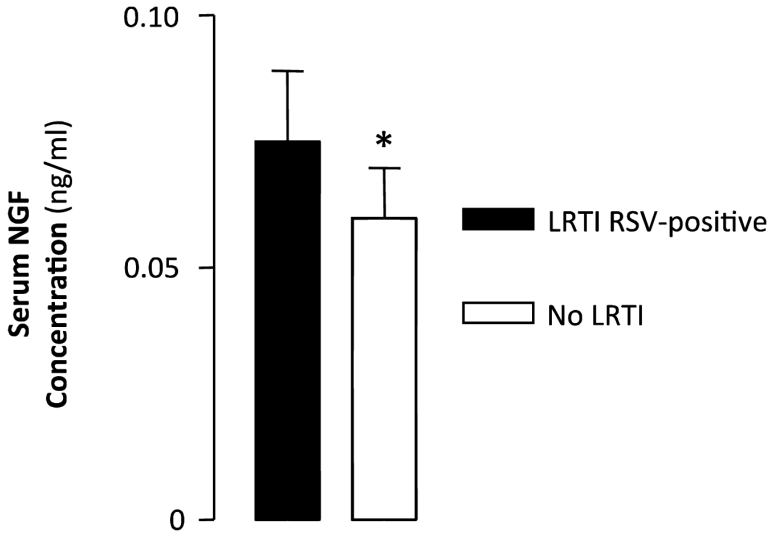

Figure 2. Blood NGF in RSV bronchiolitis

Bar graph shows NGF concentrations in the blood drawn at 72 hours post-intubation from 6 infants with RSV-positive LRTI and 6 RSV-negative post-surgical infants without LRTI. Blood samples drawn from the same patients at earlier time points showed no statistical significance. *P < 0.05.

Table #1

Neurotrophins as Biomarkers of Airway Disease

Neurotrophin	Cognate Receptor	Changes with Airway Disease
Nerve Growth Factor (NGF)	TrkA $p75^{NTR}$	- Elevated NGF and TrkA expression in human airways during RSV LRTI - NGF prevents apoptosis of RSV infected cells and keeps them alive to allow completion of viral life cycle - Airway hyperreactivity is NGF and TrkA-dependent in animal models of horizontal and vertical RSV infection - Elevated serum NGF in human asthmatics after allergen challenge - Elevated NGF in the airways of humans with active pulmonary sarcoidosis - Strong NGF and TrkA expression in lung cancer
Brain-derived Neurotrophic Factor (BDNF)	TrkB $p75^{NTR}$	- Elevated airway BDNF expression in human airways during RSV LRTI - Elevated serum BDNF and TrkB expression in asthmatics after allergen challenge - Weak BDNF and TrkB expression in lung cancer

Table #2

Extrapulmonary RSV Infection

Extrapulmonary Site of RSV Detection	Biological Effects
Central Nervous System	- CSF abnormalities - Central apnea - Seizure development - Encephalopathy - Lethargy
Peripheral Nervous System	May modulate neurotransmitter synthesis/release
Bone Marrow Stroma	- De-polymerization of actin cytoskeleton - Inhibition of pro-B cell maturation - Synthesis of pro-inflammatory cytokines (IL-6) - Inhibition of TGF-β synthesis
Peripheral Blood Mononuclear Cells	- May influence innate immune response - RSV RNA level in PBMCs correlate with severity
Myocardium	Sino-atrial blockade
Liver	- Elevation of serum liver enzymes - Development of hepatitis
Middle Ear	Otitis media

Table #3

Immunological Biomarkers of RSV Infection

Cytokine	Effect of RSV Infection	Immune Cell	Effect of RSV Infection
IL-A	Elevated with Th2 polarization by RSV LRTI	**Dendritic Cells**	Increased in bronchiolar airways
IL-5	Elevated with Th2 polarization by RSV LRTI	**Eosinophils**	Infiltration of bronchiolar airways
IL-13	Elevated withTh2 polarization by RSV LRTI	**Neutrophils**	Infiltration of bronchiolar airways
TNF-α	Reduced plasma level correlated with hospitalization	**Macrophages**	Infiltration of bronchiolar airways
IFN-γ	- Reduced in NP secretions during RSV LRTI - Reduced plasma level correlated with mechanical ventilation requirement	**$CD4^+$ T cells**	- Infiltration of bronchiolar airways - Blood count correlated with mechanical ventilator requirements
IL-8	- Elevated in plasma during RSV LRTI - Elevated plasma level correlated with mechanical ventilation requirement	**CDS* T Cells**	- Infiltration of bronchiolar airways - Reduced cytotoxic function due to increased Th2 cytoldnes - Reduced activation, proliferation, and function due to RSV cytotoxicity
IL-6	- Elevated in TAP during severe RSV LRTI - Reduced plasma level correlated with increased infant hospitalization	**Peripheral Blood Mononuclear Cells**	Proliferation associated with increased infant ICU admissions
CCL5 (RANTES)	- Reduced in plasma during RSV LRTI - Plasma level correlated with disease severity		
IL-17	Elevated in TAP during severe RSV LRTI		

Table #4

Other Potential Biomarkers of RSV Bronchiolitis

Potential Blomarker	Findings During RSV Bronchiolitis
Cysteinyl Leukotrienes	- Elevated nasal LTC_4 levels during acute RSV infection correlate with lower airway involvement and clinical severity - Urine metabolite LTE_4 elevated in infants with RSV bronchiolitis - Higher urinary LTE_4 in children with younger age or genetic predisposition to develop atopy - Not affected by extrinsic environmental factors
Lactate Dehydrogenase (LDH)	Concentration within nasal washings predicts admissions from ER with 88% accuracy
Blood-borne Biomarkers	- Decreased serum Na^+ and elevated antidiuretic hormone (ADH) observed during RSV bronchiolitis vs. RSV infection of the upper respiratory tract - RSV viral load correlates with clinical severity - RSV RNA levels can be detected in circulating PBMCs as a potential blomarker of clinical severity
Patient-Specific Gene Expression Profiling	- Specific host gene expression patterns in the nasopharynx during RSV infection may predict clinical severity - Whole-blood RNA sequencing profiles of infected patients positively identify viral strain with 95% accuracy and may predict clinical severity