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Interleukin and neurotrophin up-regulation correlates with severity of H1N1 infection in children: a case–control study

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SUMMARY

Objective: To evaluate the correlation between cytokine and neurotrophin expression and clinical findings, disease severity, and outcome of children with H1N1 influenza infection.
Methods: A prospective observational clinical study was performed on 15 children with H1N1 infection, 15 controls with lower respiratory tract infections (LRTI), and 15 non-infected children. Plasma levels of interleukin (IL)-1β, IL-6, and neurotrophic factor (nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF)) were measured using immunoenzymatic assays.
Results: Significantly higher levels of IL-1β, IL-6, BDNF, and NGF were detected in patients with H1N1 infection compared to LRTI controls, while there was no significant variation in GDNF in the two groups. IL-1β, IL-6, BDNF, and NGF levels were significantly higher in H1N1 patients with more severe clinical manifestations compared to H1N1 patients with mild clinical manifestations. Of note, IL-6 was significantly correlated with the severity of respiratory compromise and fever, while NGF up-regulation was associated with the duration of cough. No correlation was found between interleukin and neurotrophic factor expression and outcome.
Conclusions: H1N1 infection induces an early and significant IL-1β, IL-6, BDNF, and NGF up-regulation. The over-expression of these molecular markers is likely to play a neuroimmunomodulatory role in H1N1 infection and may contribute to airway inflammation and bronchial hyper-reactivity in infected children.

1. Introduction

In recent years the world has faced a new pandemic caused by an H1N1 influenza virus that contains a unique combination of gene segments never before identified in humans or animals.[1] This new pandemic strain is of particular concern because of its efficient person-to-person transmission, responsible for increased virulence and morbidity in humans.[2]

Infection with the H1N1 virus was identified as a cause of febrile respiratory infections ranging from self-limited to severe illness in both adults and children. A small percentage of children develop more severe symptoms, such as elevated fever, persistent cough, pneumonia, and acute respiratory distress syndrome (ARDS),[3,4] requiring admission to the pediatric intensive care unit (PICU) and mechanical ventilation.[5]

Several hypotheses to explain this particular virulence of H1N1 in children have been advocated, including down-regulation of type 1 interferon expression, apoptosis, and hyperinduction of proinflammatory cytokines.[6] Increased biosynthesis of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-10, and a cytokine-mediated inflammatory response, has also been documented as responsible for the severity of different viral lung infections, by increasing vascular permeability and leukocyte accumulation in lung tissue.[7–9]

Studies conducted in experimental animal models suggest that up-regulation of neurotrophins also plays a key role in the inflammatory host response during viral lung infections.[10] In particular, respiratory syncytial virus (RSV) determines an

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increased expression of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) in infected lungs.[11] In contrast to RSV infection, data regarding neurotrophin responses in other types of influenza virus infection are limited. These studies have produced contrasting results because both loss in hippocampal expression of neurotrophic factors and NGF up-regulation in the mouse model of influenza virus infection have been reported.[12,13]

NGF acts on the nociceptive fibers innervating the lower respiratory tract and increases the biosynthesis of proinflammatory neurotransmitter substance P,[14] and its high-affinity receptor (neurokinin 1) on inflammatory cells, leading to enhanced neurogenic inflammation in infected lungs.[15,16] NGF is also involved in the sensitization of spinal circuitry that underlies many forms of bronchiolar hyper-responsiveness, cough, and asthma.[17–19]

BDNF is a neuropeptide involved in the central inflammatory host response related to influenza virus infection.[20] BDNF, together with NGF, has been shown to be up-regulated in the lung cells following chemical injury and virus-mediated inflammation, both in experimental animal models and in infants with RSV infection.[21–23]

However, the role of most cytokines and neurotrophins in relation to clinical findings, disease severity, and the outcome of patients with H1N1 virus infection has thus far remained unclear. In an attempt to clarify their role in this infection and to better characterize the blood profile of these neurotrophins and cytokines, we evaluated the plasma levels of IL-1β, IL-6, NGF, BDNF, and glial derived neurotrophic factor (GDNF) in 15 children with H1N1 infection, 15 controls with lower respiratory tract infections (LRTI), and 15 non-infected children to determine whether a correlation with the expression of these molecular markers and clinical findings of patients exists.

2. Materials and methods

2.1. Study population

A prospective observational clinical study was conducted among children admitted with a diagnosis of influenza H1N1 infection or LRTI to our pediatric department in Rome, Italy, from October 2009 to December 2010. Patients with H1N1 influenza virus infection were grouped according to age, chest radiograph findings, clinical characteristics, respiratory care, and final outcome (Table 1). We also decided to group these patients into two subgroups based on the severity of their symptoms: those with severe manifestations of H1N1 infection and those with mild manifestations. We considered severe manifestations of H1N1 infection to be the presence of hypoxia at admission (SpO_2 less than 80% in room air), ARDS requiring mechanical ventilation or non-invasive ventilation (NIV) by helmet, oxygen supplementation by Ventimask or continuous positive airway pressure (CPAP) by face-mask, severity of fever (>39 °C at admission), duration of cough, and, finally, the presence of specific radiological findings, such as pneumothorax, pneumopericardium, and pneumomediastinum (Table 1). Based on these admission parameters, nine patients with severe manifestations of H1N1 influenza virus infection were admitted to the PICU, while the other six patients with mild symptoms of the disease were admitted to the pediatric infectious disease unit (PIDU). Regarding the LRTI patients, eight infants with severe RSV bronchiolitis were admitted to the PICU, while the other seven children were admitted to the PIDU. Six infants with RSV bronchiolitis admitted to the PICU underwent oxygen supplementation and NIV by helmet, while the other two patients required mechanical ventilation. The other seven infants belonging to the control group required only symptomatic treatment (Table 2).

Oral oseltamivir (60 mg twice daily for 5 days) was administered to all 15 patients with a diagnosis of influenza H1N1 and supportive therapy was started based on the severity of respiratory failure. All patients were isolated at admission based on their clinical symptoms, suspected of H1N1 infection or another acute respiratory illness. Throat/nose swabs and blood samples were taken at admission, before the start of any treatment, for both laboratory studies and cytokine/neurotrophic factor determination. All throat and nose swabs were sent to the microbiology laboratory for detection (by culture and molecular tests) of influenza A, B, and subtypes of A by influenza real-time reverse transcriptase (RT)-PCR test, and for the detection of RSV, adenovirus, parainfluenza viruses, and human metapneumovirus infections; culture alone was used for the detection of bacteria. The study was approved by the institutional review board, and the parents of participating children were informed regarding the study and provided written informed consent.

2.2. Statistical analysis

The non-parametric Mann–Whitney test and *t*-test were used to perform statistical comparisons of continuous variables between the children with H1N1 infection and the control group. Analysis of variance was performed using the Tukey–Kramer test to compare levels of IL-6, IL-1β, BDNF, NGF, and GDNF in the studied population. Linear regression analysis was used to evaluate the correlation between interleukin and neurotrophin expression and clinical manifestations in H1N1 patients. The coefficient of determination (R^2) was taken as a measure of the goodness of fit of the model. A p-value of <0.05 was considered significant. Statistical and database software used were GraphPad version 5.0 (GraphPad Software, San Diego, CA, USA) and Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA), respectively.

3. Results

3.1. Clinical and laboratory differences between H1N1 patients, LRTI controls, and 'healthy children'

Fifteen patients with H1N1 virus infection, 15 controls with LRTIs, and 15 non-infected children, defined as 'healthy children', were included in this study (see information on plasma sample collection in the **Supplementary Material**). Patients with H1N1 infection were aged 1.1 to 4.3 years, with a mean age of 2.93 years; LRTI controls were aged 11 months to 3.70 years, with a mean age of 1.93 years; 'healthy children' were aged 14 months to 3.2 years, with a mean age of 2.61 years ($p = 0.36$). Nine children with severe H1N1 virus infection were admitted to our PICU due to the severity of their respiratory compromise, while the other six patients were admitted to the PIDU. Among the children with LRTIs, eight of 15 were admitted to the PICU with a diagnosis of severe RSV bronchiolitis, while the other seven were admitted to the PIDU (four with a diagnosis of non-RSV bronchiolitis and three with a diagnosis of influenza A (H3N2) virus infection). Regarding clinical differences between the two groups, H1N1 patients experienced higher median fever (39.2 °C) compared to controls (37.7 °C) ($p < 0.0001$). Cough was a common symptom in both groups. However, H1N1 patients more frequently suffered from a dry and longer cough compared to LRTI patients (median 6 days vs. 4 days; $p < 0.0001$). The most frequent pulmonary abnormalities on chest X-ray in the H1N1 patients were represented by pneumonia and pulmonary consolidation, while in LRTI children we detected atypical findings, such as hyperinflated lungs and segmental pulmonary atelectasia. Two patients with H1N1 infection showed pneumothorax, while another three children showed severe

Table 1
Patient demographic and clinical characteristics and respiratory care and complications in H1N1-infected children

Patient	Age, years	Fever at first day of admission, °C	Duration of cough, days	SpO_2 at admission in room air (%)	Chest X-ray	Respiratory care	Antimicrobial therapy	Complications	Length of stay in hospital, days	Outcome
1	3.3	39.6 ± 0.5	8	78	Pneumothorax	O_2 supplementation by Ventimask	Ceftriaxone, clarithromycin, oseltamivir	None	11	Good
2	2.5	39.7 ± 0.4	6	78	Pneumonia	CPAP by helmet (1 day) + O_2 by Ventimask	Ceftriaxone, oseltamivir	None	10	Good
3	3.6	39.1 ± 0.6	8	70	Interstitial pneumonia, pleural effusion	NIV by full-face mask, EI/MV (2 days), CPAP by helmet (4 days)	Ceftriaxone, clarithromycin, oseltamivir	Pneumorrhachis	19	Good
4	2.1	39.3 ± 0.6	6	80	Pneumonia	O_2 supplementation by Ventimask	Ceftriaxone, oseltamivir	None	9	Good
5	4.3	39.4 ± 0.7	7	79	Bilateral pulmonary infiltrates	O_2 supplementation by Ventimask	Ceftriaxone, oseltamivir,	None	9	Good
6	3.4	39.2 ± 0.4	7	50	Bilateral pulmonary consolidation	EI/MV (11 days), CPAP by helmet (4 days)	Ceftriaxone, clarithromycin, oseltamivir	None	27	Good
7	2.8	39.7 ± 0.6	6	76	Bilateral pulmonary infiltrates	O_2 supplementation by Ventimask	Ceftriaxone, oseltamivir	Seizures	11	Good
8	1.1	39.2 ± 0.3	5	75	Pneumonia	O_2 supplementation by Ventimask	Ceftriaxone, oseltamivir	Pneumopericardium	18	Good
9	4.3	39.5 ± 0.4	9	73	Pneumothorax, bilateral pulmonary consolidation	NIV by full- face mask	Ceftriaxone, oseltamivir	None	18	Good
10	2.4	38.5 ± 0.5	7	90	Interstitial pneumonia	O_2 supplementation	Clarithromycin, oseltamivir	None	7	Good
11	2.3	38.7 ± 0.2	4	92	Normal	O_2 supplementation	Oseltamivir	None	4	Good
12	3.8	38.2 ± 0.4	5	94	Normal	None	Oseltamivir	None	3	Good
13	3.3	38.3 ± 0.7	5	95	Normal	None	Oseltamivir	None	3	Good
14	1.9	38.4 ± 0.6	6	91	Hyperinflated lung	O_2 supplementation	Oseltamivir	None	6	Good
15	1.5	38.1 ± 0.4	6	93	Normal	None	Oseltamivir	None	3	Good

CPAP, continuous positive airway pressure; EI, endotracheal intubation; MV, mechanical ventilation; NIV, non-invasive ventilation.

Table 2
Patient demographic and clinical characteristics and respiratory assessment and complications in LRTI children

Patient	Age, years	Fever at first day of admission, °C	Duration of cough, days	SpO_2 at admission in room air (%)	Chest X-ray	Respiratory care	Antimicrobial therapy	Complications	Length of stay in hospital, days	Outcome
1	1.5	37.4 ± 0.3	4	74	Segmental pulmonary atelectasia	CPAP by helmet and O_2 supplementation	Amoxicillin	None	7	Good
2	0.11	37.5 ± 0.2	5	72	Segmental pulmonary atelectasia and hyperinflated lung	Mechanical ventilation and O_2 supplementation	Amoxicillin, ceftriaxone	None	6	Good
3	1.6	38.1 ± 0.1	5	75	Hyperinflated lung	O_2 supplementation by face mask	Amoxicillin	None	5	Good
4	1.1	38.3 ± 0.2	3	71	Hyperinflated lung	CPAP by helmet and O_2 supplementation	Amoxicillin	None	5	Good
5	1.3	37.4 ± 0.4	4	80	Segmental pulmonary atelectasia	CPAP by helmet and O_2 supplementation	Amoxicillin	None	5	Good
6	1.4	37.2 ± 0.6	3	78	Hyperinflated lung	CPAP by helmet and O_2 supplementation	Amoxicillin	None	5	Good
7	1.8	38.5 ± 0.6	4	70	Hyperinflated lung	Mechanical ventilation and O_2 supplementation	Clarithromycin, amoxicillin	None	7	Good
8	1.1	38.1 ± 0.3	5	75	Segmental pulmonary atelectasia	CPAP by helmet and O_2 supplementation	Amoxicillin	None	5	Good
9	3.10	37.5 ± 0.2	4	91	Segmental pulmonary atelectasia	O_2 supplementation	Amoxicillin	None	3	Good
10	3.4	37.5 ± 0.3	3	91	Interstitial pneumonia	O_2 supplementation	Clarithromycin	None	3	Good
11	3.7	37.7 ± 0.5	4	88	Normal	O_2 supplementation	Amoxicillin	None	4	Good
12	2.3	37.5 ± 0.3	3	93	Normal	None	Amoxicillin	None	2	Good
13	2.3	38.1 ± 0.4	3	90	Normal	O_2 supplementation	Amoxicillin	None	3	Good
14	1.2	38.3 ± 0.6	3	92	Hyperinflated lung	O_2 supplementation	Amoxicillin	None	3	Good
15	3.1	37.8 ± 0.5	4	93	Normal	None	Amoxicillin	None	2	Good

LRTI, lower respiratory tract infection; CPAP, continuous positive airway pressure.

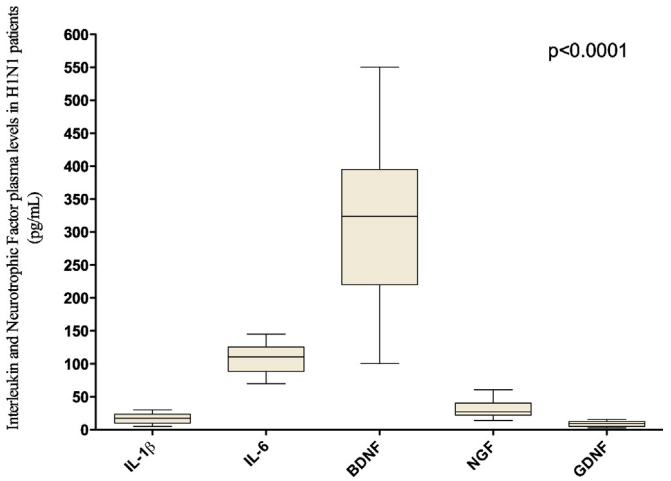

Figure 1. Box plot showing the plasma levels of IL-1β, IL-6, BDNF, NGF, and GDNF in H1N1 patients. The levels of IL-6 and BDNF were significantly higher compared to the other biomarkers evaluated ($p < 0.0001$).

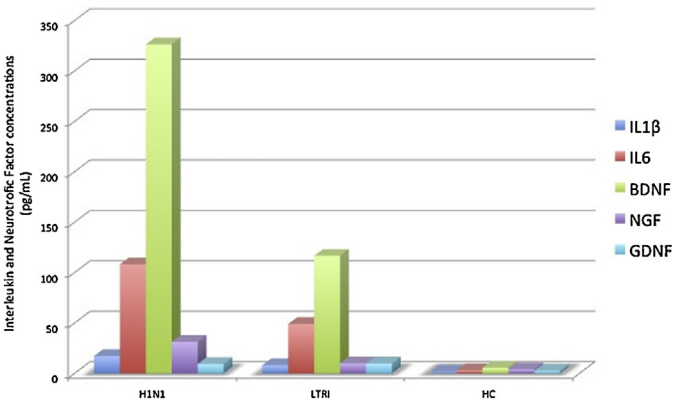

Figure 2. Histogram showing the levels of IL-1β, IL-6, BDNF, NGF, and GDNF in H1N1 patients (H1N1), LRTI controls (LRTI), and in 'healthy children' (HC).

respiratory complications, such as pneumopericardium, pneumomediastinum, and pneumorrhachis on chest computed tomography scan (Table 1). No pulmonary or systemic complications were found in the LRTI group. No differences in clinical manifestations, such as gastrointestinal and neurological symptoms, were found between the two groups. Regarding laboratory tests (blood cell and platelet counts, serum C-reactive protein, procalcitonin, alanine aminotransferase, aspartate aminotransferase, creatinine, urea) no significant differences were detected between H1N1 patients and LRTI controls. All children, both patients and controls, had a good outcome without any significant complications, however H1N1 patients had a significantly longer duration of hospitalization compared to the control group (median 9 days vs. 3 days; $p = 0.0013$).

3.2. Interleukin and neurotrophic factor expression in H1N1 patients, LRTI controls, and 'healthy children'

We detected different plasma levels of both interleukins and neurotrophic factors in H1N1 patients. In these patients we found significantly ($p < 0.0001$) higher levels of IL-6 (108.1 ± 22.8 pg/ml) compared to IL-1β (17.2 ± 7.9 pg/ml) and BDNF (326.3 ± 133.3 pg/ml) compared to NGF (31.2 ± 13.6 pg/ml) and GDNF (9.3 ± 4.1 pg/ml) (Figure 1). Also, in LRTI patients, the mean plasma levels of IL-6 were significantly higher compared to the levels of IL-1β (49.0 ± 10.3 pg/ml vs. 8.1 ± 2.2 pg/ml) ($p < 0.0001$), while the mean plasma levels of BDNF were significantly higher compared to the levels of both NGF and GDNF (BDNF 116.6 ± 28.0 pg/ml; NGF 9.9 ± 3.0 pg/ml; GDNF 9.8 ± 3.3 pg/ml; $p < 0.0001$). Differently from H1N1 patients and LRTI controls, we found no significant differences between plasma levels of interleukins and neurotrophins in 'healthy children'. In these children, the mean plasma levels of IL-6 and IL-1β were 3.1 ± 0.3 pg/ml and 1.6 ± 0.2 pg/ml, respectively ($p = 0.49$), while the levels of BDNF, NGF, and GDNF were 5.8 ± 2.6 pg/ml, 4.0 ± 2.1 pg/ml, and 3.5 ± 2.5 pg/ml, respectively ($p = 0.52$) (Figure 2). No significant correlations were found between interleukin and neurotrophic factor expression and the age of H1N1 patients, LRTI controls, and 'healthy children' (data not shown).

3.3. Plasma level differences in interleukin and neurotrophic factor expression between H1N1 patients and controls

Significantly higher levels of interleukin IL-6, IL-1β, BDNF, and NGF were demonstrated in all patients with H1N1 infection

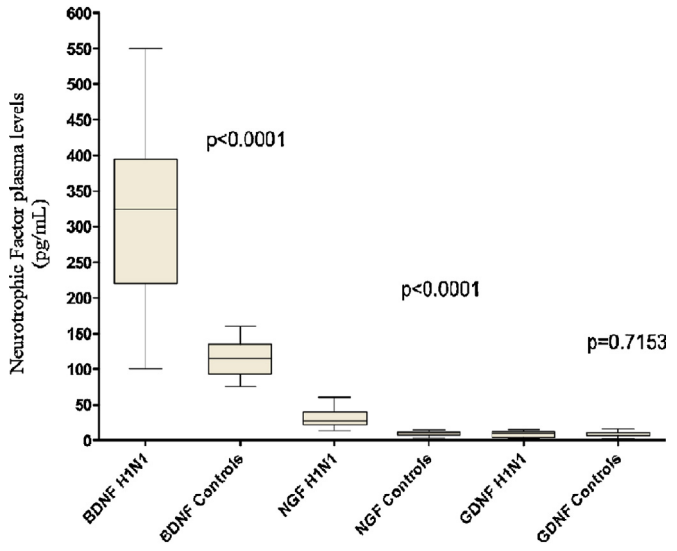

Figure 3. Box plot showing the plasma levels of neurotrophins in patients and in controls. H1N1 patients showed significantly higher levels of BDNF ($p < 0.0001$) and NGF ($p < 0.0001$) compared to LRTI controls. There were no significant differences in GDNF levels between the two groups ($p = 0.7153$).

compared to the controls, while GDNF levels did not vary significantly between the two groups. Compared with LRTI patients, H1N1 patients displayed significantly higher plasma levels of IL-6 (108.1 ± 22.8 pg/ml vs. 49.0 ± 10.3 pg/ml; $p < 0.0001$) and IL-1β (17.2 ± 7.9 pg/ml vs. 8.1 ± 2.2 pg/ml; $p < 0.0002$) (data not shown). As shown in Figure 3, BDNF and NGF plasma levels were also significantly higher in H1N1 patients compared to children with LRTIs. In H1N1 patients, the mean level of BDNF was 326.3 ± 133.3 pg/ml, while in LRTI children, the BDNF mean level was 116.6 ± 28.0 pg/ml ($p < 0.0001$). With regard to NGF levels, significant differences were detected between the two groups: in H1N1 patients, the mean NGF level was 31.2 ± 13.6 pg/ml, while in LRTI children, the mean NGF level was 9.9 ± 3.0 pg/ml ($p < 0.0001$) (Figure 3). No significant changes were observed in the GDNF plasma level between the two groups (9.3 ± 4.1 pg/ml in H1N1 patients compared to 9.8 ± 3.3 pg/ml in LRTI patients; $p = 0.71$).

3.4. Correlation between interleukin and neurotrophic factor expression and disease severity and clinical manifestations in H1N1 patients

To elucidate the association between interleukin and neurotrophic factor expression and disease severity, we analyzed the

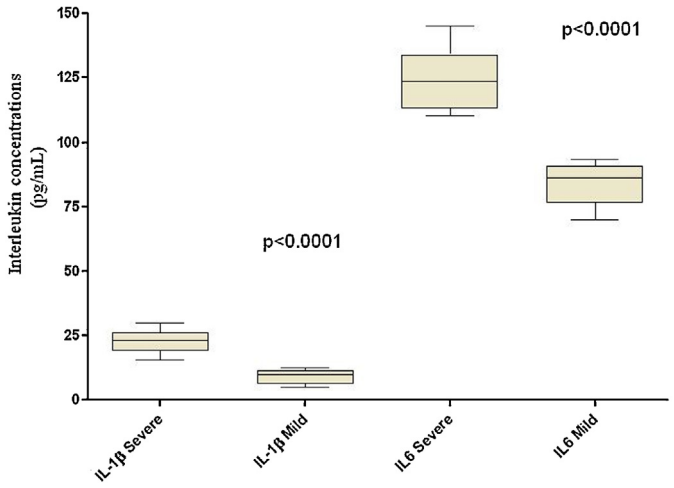

Figure 4. Box plot showing the plasma levels of interleukins in patients with H1N1 virus infections. H1N1 patients with more severe clinical manifestations of disease elicited significantly higher levels of IL-1β ($p < 0.0001$) and IL-6 ($p < 0.0001$) compared to H1N1 patients with mild symptoms.

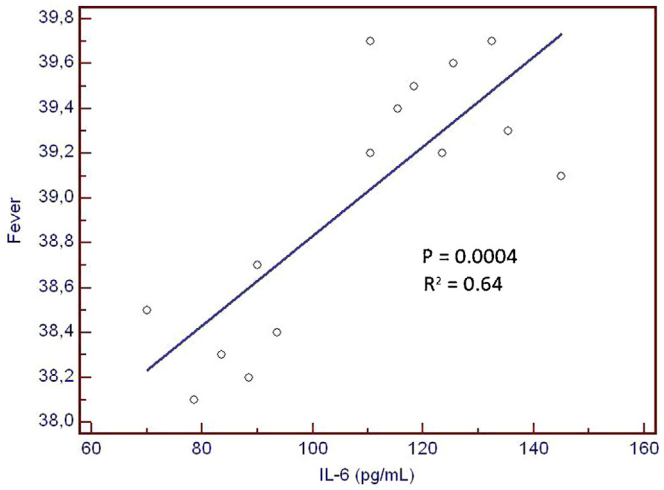

Figure 6. Scatter plot showing the significant correlation between fever and IL-6 plasma levels in H1N1 patients. The line represents the regression line (linear regression equation: fever = 0.02 × IL-6 + 36.8; $R^2 = 0.64$).

plasma levels in patients with severe symptoms (nine patients) and mild symptoms (six patients) of H1N1 influenza virus infection. Compared to the mild symptoms patients, severe symptoms H1N1 patients produced significantly higher levels of IL-1β (22.6 ± 4.7 pg/ml vs. 9.1 ± 2.8 pg/ml; $p < 0.0001$), IL-6 (124.1 ± 11.8 pg/ml vs. 84.0 ± 8.6 pg/ml; $p < 0.0001$), BDNF (404.6 ± 98.0 pg/ml vs. 208.8 ± 82.6 pg/ml; $p < 0.0015$), and NGF (38.4 ± 12.7 pg/ml vs. 20.3 ± 4.8 pg/ml; $p < 0.0058$), while no statistical differences were found in GDNF plasma levels between the two groups (8.1 ± 3.9 pg/ml vs. 11.2 ± 3.8 pg/ml, $p = 0.152$) (Figures 4 and 5).

Moreover, to verify whether there was a correlation between interleukin and neurotrophic factor up-regulation and clinical manifestations in H1N1 patients, we compared the plasma levels of these biomarkers with certain patient clinical symptoms. Of note, we detected a positive correlation between plasma levels of IL-6 and fever, with a coefficient of determination of 0.64 ($p = 0.0004$) (Figure 6). We found the same positive correlation between NGF plasma levels and the duration of cough, with a coefficient of determination of 0.37 ($p = 0.0165$) (Figure 7). Moreover, we detected the highest NGF plasma levels of all H1N1 patients in two patients with specific pulmonary complications, such as pneumothorax and pneumopericardium (60.5 pg/ml and 53.0 pg/ml, respectively). Finally, we found a negative correlation between IL-6 plasma levels and SpO_2 at admission in room air, with a coefficient of determination of 0.53 ($p = 0.020$) (Figure 8). No significant correlations were reported between interleukin and neurotrophic factor expression and other parameters evaluated, such as biochemical markers of inflammation (C-reactive protein and procalcitonin), systemic complications, and, finally, the outcome of children with H1N1 virus infection.

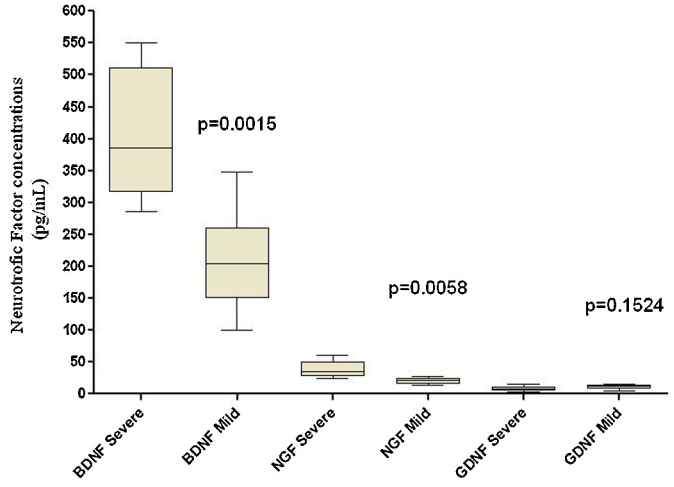

Figure 5. Box plot showing the plasma levels of neurotrophins in patients with H1N1 virus infections. H1N1 patients with more severe clinical manifestations of disease showed significantly higher levels of BDNF ($p = 0.0015$) and NGF ($p = 0.0058$) compared to H1N1 patients with mild symptoms. There were no significant differences in GDNF levels between the two groups ($p = 0.1524$).

4. Discussion

Our study, despite the limited patient sample, provides evidence that H1N1 virus infection induces significantly increased plasma levels of both interleukins and neurotrophins soon after virus lung infection, suggesting that these factors play a key role in the molecular events leading to airway inflammation and disease severity. Compared to LRTI controls and 'healthy children', H1N1-infected children showed a strong up-regulation of IL-1β, IL-6,

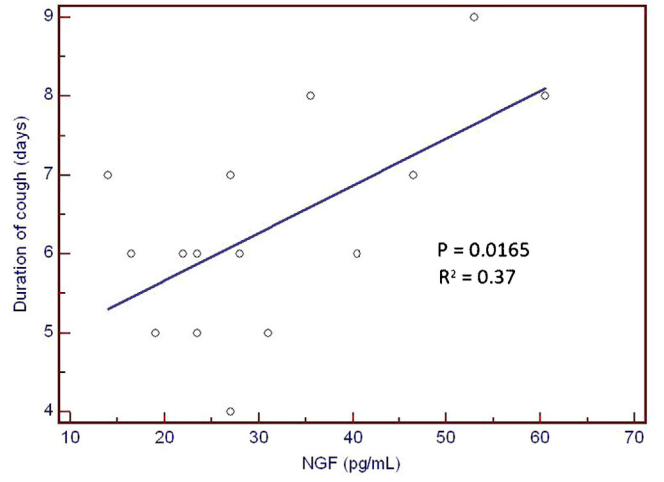

Figure 7. Scatter plot showing the significant correlation between duration of cough and NGF plasma levels in H1N1 patients. The line represents the regression line (linear regression equation: duration of cough = 0.06 × NGF + 4.5; $R^2 = 0.37$).

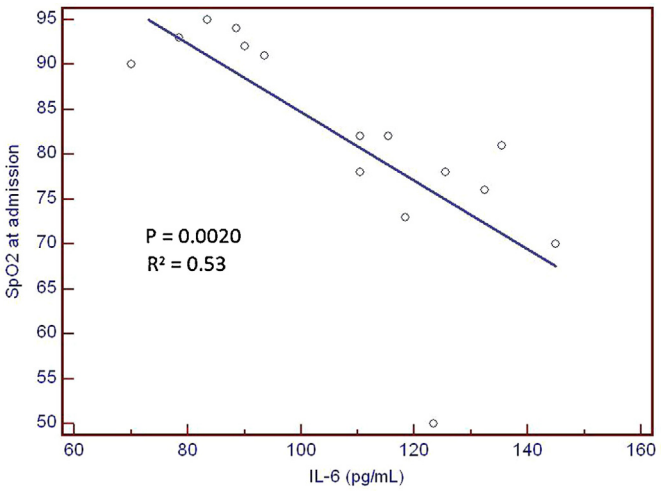

Figure 8. Scatter plot showing the significant correlation between SpO_2 at admission in room air and IL-6 plasma levels in H1N1 patients. The line represents the regression line (linear regression equation: $SpO_2 = -0.38 \times \text{IL-6} + 122.9$; $R^2 = 0.53$).

BDNF, and NGF, which was correlated with the severity of clinical compromise assessed upon admission, while no differences were detected in GDNF expression in the two patient groups. We also observed that in H1N1 patients with more severe clinical manifestations of disease, plasma levels of these interleukins and neurotrophins were significantly increased compared to those of H1N1 patients with mild symptoms, and that this up-regulation was correlated with some specific clinical manifestations and a longer duration of hospitalization. In particular, IL-6 was significantly correlated with the severity of respiratory compromise and higher fever, while NGF was correlated with the duration of cough in this subset of patients.

To date it has been difficult to fully elucidate the role of interleukins and neurotrophins in the mechanisms of the virus–host response, because both proinflammatory and immunoprotective actions have been reported. H1N1 virus infection causes the activation of the host macrophages and lymphocytes determining the release of proinflammatory cytokines and neuropeptides.[6] The increased biosynthesis of proinflammatory cytokines in the lung tissue may lead to higher blood vessel permeability, phagocytic cell recruitment, apoptosis of lung epithelial cells, and the release of neutrophil-derived enzymes, such as myeloperoxidase and elastase, responsible for the severity of acute lung injury after virus infection.[24,25]

Cytokine up-regulation may also cause epithelial cell damage by increasing the production of nitric oxide synthase and cyclooxygenase, and by favoring the release of the excitatory amino acids, as found in an experimental model of pulmonary edema.[26,27]

Previous studies have reported the correlation between IL-1β and IL-6 up-regulation and certain clinical and radiological findings, such as fever, pneumonia, and ARDS, both in experimental animal models and in children with naturally acquired seasonal influenza A.[25,26,28] In particular, IL-1β and IL-6 have been identified as specific markers of the severity of acute lung injury during H1N1 influenza virus infection,[7] and it has also been reported that IL-1β is an early and useful marker of the severity and progression of lung inflammation in patients undergoing mechanical ventilation and unresponsive to antimicrobial treatment.[29] Our results are consistent with those of previous research, because children with more severe clinical manifestations of H1N1 infection elicited a more intensive production of IL-1β and IL-6 than H1N1 patients with mild symptoms, suggesting that this up-regulation exerted a key role in the development of lung inflammation.[30,31]

Our results also show that H1N1 patients produced higher levels of BDNF and NGF than LRTI controls, confirming the role of these neurotrophins in the inflammatory host response after H1N1 infection. Of note, we also observed that in H1N1 patients with more severe clinical manifestations of disease, plasma levels of both these neurotrophins were significantly increased compared to those in H1N1 patients with mild symptoms, and that this up-regulation was correlated with specific clinical manifestations, such as the severity of pulmonary compromise and cough.

NGF, through its multiple actions on the airways and immune and inflammatory cells, plays a key role in bronchiolar hyper-responsiveness and cough.[11,19,22,32] Recent studies have reported a significant correlation between cough and asthma and increased NGF sputum levels, suggesting a strong relationship between NGF up-regulation and the severity of airway hyper-responsiveness.[33,34] It has also been reported that NGF enhances the contractile responses of tracheal strips to histamine and neurokinin A, both in experimental animal models and in humans with viral lung infections, confirming that this neurotrophin, either alone or synergistically with BDNF, represents an essential link between viral-infected epithelial cells and the sub-epithelial neural networks.[6,18,23,35,36] Our results are in keeping with this evidence, because children with a longer duration of cough and with specific pulmonary complications, such as pneumothorax and pneumopericardium, elicited higher NGF and BDNF plasma levels involved in the development of bronchial hyper-reactivity, lung inflammation, and cough after H1N1 virus infection.

Until recently it has been difficult to explain whether the observed NGF and BDNF up-regulation in children with H1N1 virus infection represents innate protective mechanisms for respiratory cell survival or is secondary to a loss of physiological control of neurotrophin/neurotrophin receptor biosynthesis. It is possible that the biosynthesis of these neurotrophins occurs at the level of infected structural cells of the respiratory airways, as well as inflammatory cells stimulated by the H1N1 virus, such as activated CD4 T lymphocytes.[37,38] Available clinical and experimental data do not permit a definitive clarification of these findings. Neurotrophin plasma levels increase in several inflammatory diseases, whereas up-regulation of TrkA/TrkB receptors has been shown following different inflammatory stimuli, such as allergen provocation and asthma. Recently lymphocytes, and in particular activated T-cells, have been revealed to express BDNF and BDNF receptors in the experimental animal model of pulmonary sarcoidosis and chemical lung injury.[39,40] So, it is possible that neurotrophin up-regulation is secondary to rapid lymphocyte activation by H1N1 virus infection and that this over-expression represents an important process in the mechanisms of the inflammatory host response after viral lung infections.[23]

In fact, previous studies have reported that different viral lung infections are associated with early neurotrophin biosynthesis, mainly BDNF and neurotrophin-3 (NT-3), suggesting that the changes in neurotrophin release may contribute to the development of lung inflammation and airway remodeling.[23] In our study, NGF and BDNF up-regulation, observed early after H1N1 virus infection, is consistent with the timing of neurotrophin expression found in an experimental model of virus-infected human alveolar macrophages, suggesting that these neurotrophins act in a different fashion to amplify and propagate inflammation in infected airways.[41]

In conclusion, our observations provide new evidence that a comprehensive neuroimmune response is activated at an early stage in pandemic H1N1 influenza virus infection, with up-regulated production of plasma interleukins IL-1β and IL-6 and neurotrophins NGF and BDNF. These findings are consistent with

previous experimental and clinical studies, confirming a key role for both interleukins and neurotrophins in the pathogenesis of airway inflammation and hyper-reactivity during viral lung infections. The increased expression of interleukins and neurotrophins may together be the underlying biochemical cause of the observed clinical symptoms in H1N1 patients with severe symptoms, and defining the relationships between interleukin and neurotrophic factor expression and the pathophysiology of H1N1 may help to shed light on the molecular aspects of H1N1 and other human viral lung infections. Further clinical and experimental investigations are necessary to identify the interleukin and neurotrophin target cells in the damaged lung and to discover possible clinical applications of these molecular markers in children with H1N1 and other viral lung infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2013.07.006.

References

1. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009;**325**:197–201.
2. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* 2009;**460**:1021–5.
3. Hasegawa M, Hashimoto K, Morozumi M, Ubukata K, Takahashi T, Inamo Y. Spontaneous pneumomediastinum complicating pneumonia in children infected with the 2009 pandemic influenza A (H1N1) virus. *Clin Microbiol Infect* 2010;**16**:195–9.
4. Kamigaki T, Oshitani H. Epidemiological characteristics and low case fatality rate of pandemic (H1N1) 2009 in Japan. *PLoS Curr* 2009;**1**:RRN1139.
5. Epidemiological summary of pandemic influenza A (H1N1) 2009 virus Ontario, Canada, June 2009. *Wkly Epidemiol Rec* 2009;**84**:485–92.
6. Yu X, Zhang X, Zhao B, Wang J, Zhu Z, Teng Z, et al. Intensive cytokine induction in pandemic H1N1 influenza virus infection accompanied by robust production of IL-10 and IL-6. *PLoS One* 2011;**6**:e28680.
7. Estella A. Cytokine levels in bronchoalveolar lavage and serum in 3 patients with 2009 influenza A(H1N1)v severe pneumonia. *J Infect Dev Ctries* 2011;**5**:540–3.
8. Maruo N, Morita I, Shirao M, Murota S. IL-6 increases endothelial permeability in vitro. *Endocrinology* 1992;**131**:710–4.
9. Julkunen I, Sareneva T, Pirhonen J, Ronni T, Melén K, Matikainen S. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev* 2001;**12**:171–80.
10. Piedimonte G. Contribution of neuroimmune mechanisms to airway inflammation and remodeling during and after respiratory syncytial virus infection. *Pediatr Infect Dis J* 2003;**22**(2 Suppl):S66–74. discussion:S74–5.
11. Hu C, Wedde-Beer K, Auais A, Rodriguez MM, Piedimonte G. Nerve growth factor and nerve growth factor receptors in respiratory syncytial virus-infected lungs. *Am J Physiol Lung Cell Mol Physiol* 2002;**283**:494–502.
12. Jurgens HA, Johnson RW. Environmental enrichment attenuates hippocampal neuroinflammation and improves cognitive function during influenza infection. *Brain Behav Immun* 2012;**26**:1006–16.
13. Sheridan JF, Stark JL, Avitsur R, Padgett DA. Social disruption, immunity, and susceptibility to viral infection. Role of glucocorticoid and NGF. *Ann N Y Acad Sci* 2000;**917**:894–905.
14. Piedimonte G, Hegele RG, Auais A. Persistent airway inflammation after resolution of respiratory syncytial virus infection in rats. *Pediatr Res* 2004;**55**:657–65.
15. Piedimonte G, Rodriguez MM, King KA, McLean S, Jiang X. Respiratory syncytial virus upregulates expression of the substance P receptor in rat lungs. *Am J Physiol* 1999;**277**:L831–40.
16. King KA, Hu C, Rodriguez MM, Romaguera R, Jiang X, Piedimonte G. Exaggerated neurogenic inflammation and substance P receptor upregulation in RSV-infected weanling rats. *Am J Respir Cell Mol Biol* 2001;**24**:101–7.
17. Freund-Michel V, Frossard N. The nerve growth factor and its receptors in airway inflammatory diseases. *Pharmacol Ther* 2008;**117**:52–76.
18. El-Hashim AZ, Jaffal SM. Nerve growth factor enhances cough and airway obstruction via TrkA receptor- and TRPV1-dependent mechanisms. *Thorax* 2009;**64**:791–7.
19. de Vries A, Dessing MC, Engels F, Henricks PA, Nijkamp FP. Nerve growth factor induces a neurokinin-1 receptor-mediated airway hyperresponsiveness in guinea pigs. *Am J Respir Crit Care Med* 1999;**159**:1541–4.
20. Braun A, Appel E, Baruch R, Herz U, Botchkarev V, Paus R, et al. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. *Eur J Immunol* 1998;**28**:3240–51.
21. Jurgens HA, Amancherla K, Johnson RW. Influenza infection induces neuroinflammation, alters hippocampal neuron morphology, and impairs cognition in adult mice. *J Neurosci* 2012;**32**:3958–68.
22. Urrego F, Scuri M, Auais A, Mohtasham L, Piedimonte G. Combined effects of chronic nicotine and acute virus exposure on neurotrophin expression in rat lung. *Pediatr Pulmonol* 2009;**44**:1075–84.
23. Tortorolo L, Langer A, Polidori G, Vento G, Stampachiacchere B, Aloe L, et al. Neurotrophin overexpression in lower airways of infants with respiratory syncytial virus infection. *Am J Respir Crit Care Med* 2005;**172**:233–7.
24. Othumpangat S, Gibson LF, Samsell L, Piedimonte G. NGF is an essential survival factor for bronchial epithelial cells during respiratory syncytial virus infection. *PLoS One* 2009;**4**:e6444.
25. Puneet P, Moochhala S, Bhatia M. Chemokines in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 2005;**288**:L3–15.
26. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. *Chest* 1995;**108**:1303–14.
27. Chao CC, Hu S, Ehrlich L, Peterson PK. Interleukin-1 and tumor necrosis factor-alpha synergistically mediate neurotoxicity: involvement of nitric oxide and of N-methyl-D-aspartate receptors. *Brain Behav Immun* 1995;**9**:355–65.
28. Chang SS, Vaz Fragoso CA, Van Ness PH, Fried LP, Tinetti ME. Association between combined interleukin-6 and C-reactive protein levels and pulmonary function in older women: results from the Women's Health and Aging Studies I and II. *J Am Geriatr Soc* 2011;**59**:113–9.
29. Sato M, Hosoya M, Wright PF. Differences in serum cytokine levels between influenza virus A and B infection in children. *Cytokine* 2009;**47**:65–8.
30. Wu CL, Lee YL, Chang KM, Chang GC, King SL, Chiang CD, et al. Bronchoalveolar interleukin-1 beta: a marker of bacterial burden in mechanically ventilated patients with community-acquired pneumonia. *Crit Care Med* 2003;**31**:812–7.
31. Pires-Neto RC, Morales MM, Lancas T. Expression of acute-phase cytokines, surfactant proteins, and epithelial apoptosis in small airways of human acute respiratory distress syndrome. *J Crit Care* 2013;**28**:111–5.
32. Patil G, Khan MI, Patel DK, Sultana S, Prasad R, Ahmad I. Evaluation of cytotoxic, oxidative stress, proinflammatory and genotoxic responses of micro- and nanoparticles of dolomite on human lung epithelial cells A(549). *Environ Toxicol Pharmacol* 2012;**34**:436–45.
33. Chaudhuri R, McMahon AD, McSharry CP, Macleod KJ, Fraser I, Livingston E, et al. Serum and sputum neurotrophin levels in chronic persistent cough. *Clin Exp Allergy* 2005;**35**:949–53.
34. Bonini S, Lambiase A, Bonini S, Angelucci F, Magrini L, Manni L, et al. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc Natl Acad Sci U S A* 1996;**93**:10955–60.
35. Nicol GD, Vasko MR. Unraveling the story of NGF-mediated sensitization of nociceptive sensory neurons: ON or OFF the Trks. *Mol Interv* 2007;**7**:26–41.
36. de Vries A, Engels F, Henricks PA, Leusink-Muis T, McGregor GP, Braun A, et al. Airway hyper-responsiveness in allergic asthma in guinea-pigs is mediated by nerve growth factor via the induction of substance P: a potential role for trkA. *Clin Exp Allergy* 2006;**36**:1192–200.
37. Olgart Höglund C, de Blay F, Oster JP, Duvernelle C, Kassel O, Pauli G, et al. Nerve growth factor levels and localisation in human asthmatic bronchi. *Eur Respir J* 2002;**20**:1110–6.
38. Ehrhard PB, Erb P, Graumann U, Otten U. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc Natl Acad Sci U S A* 1993;**90**:10984–8.
39. Dagnell C, Grunewald J, Kramar M, Haugom-Olsen H, Elmberger GP, Eklund A, et al. Neurotrophins and neurotrophin receptors in pulmonary sarcoidosis—granulomas as a source of expression. *Respir Res* 2010;**11**:156.
40. Dagnell C, Grunewald J, Kramar M, Haugom-Olsen H, Elmberger GP, Eklund A, et al. Role of CD4(+) T cells in the modulation of neurotrophin production in mice exposed to low-level toluene. *Immunopharmacol Immunotoxicol* 2009;**31**:146–9.
41. Othumpangat S, Walton C, Piedimonte G. MicroRNA-221 modulates RSV replication in human bronchial epithelium by targeting NGF expression. *PLoS One* 2012;**7**:e30030.