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Circulating Plasma Levels of Neurotrophins are Increased in Children with H1N1 Virus Infection

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Abstract

Aim: To evaluate the correlation between neurotrophin expression and clinical findings, disease severity, and outcome of children with H1N1 viral infection.

Methods: Prospective observational clinical study performed on 15 children with H1N1 infection and 15 controls with lower respiratory tract infections. Neurotrophic factor Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), and Glial Derived Neurotrophic Factor (GDNF) plasma levels were measured using immunoenzymatic assays.

Results: Significantly higher levels of BDNF and NGF were detected in patients with H1N1 infection respect to controls, while GDNF did not show significant variations in both groups. In H1N1 patients with more severe clinical manifestations BDNF and NGF levels were significantly higher respect to H1N1 patients with mild clinical manifestations. Noteworthy, NGF up-regulation was associated with duration of cough. No correlation was found between neurotrophic factor expression and final outcome.

Conclusions: H1N1 infection induces an early and significantly BDNF and NGF up-regulation. The over-expression of these molecular markers, namely NGF, is likely to play a neuro-immunomodulatory role in H1N1 infection and may contribute to airway inflammation and bronchial hyperreactivity in infected children.

Keywords: Brain derived neurotrophic factor; Glial derived neurotrophic factor; Nerve growth factor; H1N1 virus infection

Introduction

In the last years the world has been facing a new pandemia caused by a H1N1 influenza virus which contains a unique combination of gene segments that has never been 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].

H1N1 virus infection was identified as cause of febrile respiratory infections ranging from self-limited to severe illness both in adults and children. A small percentage of children develops 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 were advocated, including down-regulation of type1 interferon expression, apoptosis and hyperinduction of proinflammatory cytokines [6]. Studies conducted in experimental animal models suggest that also up-regulation of neurotrophins plays a key role in the inflammatory host response during viral lung infections [7]. In particular, Respiratory Syncytial Virus (RSV) determines an increased expression of Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF) in infected lungs [8].

NGF acts on the nociceptive fibers innervating the lower respiratory tract and increases the biosynthesis of proinflammatory neurotransmitter substance P, and its high-affinity receptor (neurokinin 1) on inflammatory cells, leading to enhanced neurogenic inflammation in infected lungs [9-11]. NGF is also involved in the sensitisation of spinal circuitry that underlies many forms of bronchiolar hyper responsiveness, cough, and asthma [12-15].

BDNF is a neuropeptide involved in the central inflammatory host response related to influenza virus infection [16]. BDNF, together with NGF, resulted up-regulated in the lung cells following chemical injury and virus-mediated inflammation both in experimental animal models and in infants with RSV infection [17-19].

The neurotrophin family includes the nerve growth factor (NGF) and other structurally related neuropeptides, such as Brain-Derived Neurotrophic Factor (BDNF) and neurotrophins 3/4 exerting a key role in the survival and development of peripheral and central nervous system neurons. NGF is synthesized in large amounts mainly in the hippocampus and cerebral cortex. It acts on forebrain cholinergic neurons located in the septum, the nucleus of the diagonal band of Broca and the nucleus basalis of Meynert. BDNF is a protein consisting of 119 amino acids produced, along with its receptor TrkB, in the hippocampus, amygdala, thalamus and cerebellum exerting its biological effects on survival and function of selected populations of dopaminergic, serotoninergic and GABAergic neurons. Plasma levels of these neurotrophins seem to be influenced by hormonal status, age and also by platelets that remain the most important predictor of their concentration [20]. Recently, it has been reported the association between neurotrophic factor levels and pediatric bipolar disorder and their role as potential molecular

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markers to guide clinical treatment decisions for psychotherapeutic interventions [21,22].

However, the role of neurotrophins in relation to clinical findings, disease severity and outcome of patients with H1N1 virus infection remains thus far unclear. Attempting to clarify their role in this infection, we evaluated the plasma levels of NGF, BDNF and GDNF in 15 children with H1N1 infection and 15 controls with Lower Respiratory Tract Infections (LRTI), to determine whether a correlation with the expression of these molecular markers and clinical findings of patients exists.

Materials and Methods

Study population

We conducted a prospective observational clinical study among children admitted from October 2009 to December 2011 with the diagnosis of influenza H1N1 infection and LRTI to our Pediatric Department in Rome, Italy. Patients with H1N1 influenza virus infection were grouped according to age, findings of chest radiograph, clinical characteristics, respiratory care, and final outcome (Table 1). We also decided to differentiate these patients in two groups (severe and mild manifestations of H1N1 infection) based on the severity of the symptoms. We considered severe manifestations of H1N1 infection 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 CPAP by face-mask, severity of fever (more than 39°C at admission), duration of cough, and, finally, 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 6 patients with mild symptoms of the disease were admitted to the PIDU. Regarding the LRTI patients, 8 infants with severe RSV bronchiolitis were admitted to the PICU, while the other 7 children to the PIDU. Six infants with RSV bronchiolitis admitted to the PICU underwent oxygen supplementation and NIV by helmet, while the other 2 patients required mechanical ventilation. The other 7 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 the diagnosis of influenza H1N1 and supportive therapy was started based on the severity of respiratory failure. All patients were isolated at the admission based on their clinical symptoms suspected for H1N1 infection or other acute respiratory illness. The throat/nose swabs and blood samples for both laboratory studies and cytokines/neurotrophic factor determination were taken at the admission. All the throat/nose swabs were sent to the microbiology for influenza virus detection and were analyzed for influenza A, B, subtypes of A by influenza real-time RT-PCR test, and for RSV infection. The study was approved by the institutional

Patients	Age (years)	Fever at admission	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	O2 supplementation by Ventimask	Ceftriaxone, Clarithromycin Oseltamivir	None	11	Good
2	2.5	39.7 ± 0.4	6	78	Pneumonia	CPAP by Helmet (1 day) + O2 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	Pneumorrachis	19	Good
4	2.1	39.3 ± 0.6	6	80	Pneumonia	O2 supplementation by Ventimask	Ceftriaxone Oseltamivir	None	9	Good
5	4.3	39.4 ± 0.7	7	79	Bilateral pulmonary infiltrates	O2 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	O2 supplementation by Ventimask	Ceftriazone Oseltamivir	Seizures	11	Good
8	1.1	39.2 ± 0.3	5	75	Pneumonia	O2 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	O2 supplementation	Clarithromycin Oseltamivir	None	7	Good
11	2.3	38.7 ± 0.2	4	92	Normal	O2 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	O2 supplementation	Oseltamivir	None	6	Good
15	1.5	38.1 ± 0.4	6	93	Normal	None	Oseltamivir	None	3	Good

Abbreviations: CPAP (Continuous Positive Airway Pressure); EI (Endotracheal Intubation); MV (Mechanical Ventilation).

Table 1: Demographic and Clinical Characteristics, Respiratory Care and Complications Of H1N1 Infected Children.

Patients	Age (years)	of admission	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 O2 supplementation	Amoxicillyn	None	7	Good
2	0.11	37.5 ± 0.2	5	72	Segmental pulmonary atelectasia and hyperinflated lung	Mechanical ventilation and O2 supplementation	Amoxicillyn Ceftriaxone	None	6	Good
3	1.6	38.1 ± 0.1	5	75	Hyperinflated lung	O2 supplementation by face mask	Amoxicillyn	None	5	Good
4	1.1	38.3 ± 0.2	3	71	Hyperinflated lung	CPAP by Helmet and O2 supplementation	Amoxicillyn	None	5	Good
5	1.3	37.4 ± 0.4	4	80	Segmental pulmonary atelectasia	CPAP by Helmet and O2 supplementation	Amoxicillyn	None	5	Good
6	1.4	37.2 ± 0.6	3	78	Hyperinflated lung	CPAP by Helmet and O2 supplementation	Amoxicillyn	None	5	Good
7	1.8	38.5 ± 0.6	4	70	Hyperinflated lung	Mechanical ventilation and O2 supplementation	Clarithromycin Amoxicillyn	None	7	Good
8	1.1	38.1 ± 0.3	5	75	Segmental pulmonary atelectasia	CPAP by Helmet and O2 supplementation	Amoxicillyn	None	5	Good
9	3.10	37.5 ± 0.2	4	91	Segmental pulmonary atelectasia	O2 supplementation	Amoxicillyn	None	3	Good
10	3.4	37.5 ± 0.3	3	91	Interstitial pneumonia	O2 supplementation	Clarithromycin	None	3	Good
11	3.7	37.7 ± 0.5	4	88	Normal	O2 supplementation	Amoxicillyn	None	4	Good
12	2.3	37.5 ± 0.3	3	93	Normal	None	Amoxicillyn	None	2	Good
13	2.3	38.1 ± 0.4	3	90	Normal	O2 supplementation	Amoxicillyn	None	3	Good
14	1.2	38.3 ± 0.6	3	92	Hyperinflated lung	O2 supplementation	Amoxicillyn	None	3	Good
15	3.1	37.8 ± 0.5	4	93	Normal	None	Amoxicillyn	None	2	Good

Abbreviations: CPAP (Continous Positive Airway Pressure); EI (Endotracheal Intubation); MV (Mechanical Ventilation).

Table 2: Demographic and Clinical Characteristics, Respiratory Assessment and Complications of LRTI Children.

Review Board, and the parents of participating children were informed regarding study and provided written informed consent.

Plasma sample collection

In H1N1 patients we collected blood samples using indwelling radial artery catheters in children admitted to the PICU or arterial puncture in children admitted to the PIDU after local anesthetic ointment application. All samples were obtained in the acute phase of the illness, at the admission of the patients, and before to start any treatment. The plasma samples were submitted for microbiological and biochemical analysis (leukocyte and platelet counts, serum C-reactive protein concentration, procalcitonin, glucose-protein concentration, electrolytes, acid-base study, BUN, etc). To measure neurotrophic factor plasma levels all blood samples were centrifuged for 10 min at 5,000 rpm, and the supernatants were immediately stored at −70°C until analysis. As controls, we used blood radial artery samples collected from children with the diagnosis of LRTI who had undergone blood sample analysis at the moment of their admission to the PICU or PIDU. To better characterize the profile of these neurotrophins in the blood of children with the same age of H1N1 patients and LRTI controls, we also collected blood samples in 15 non-infected children (defined "healthy children") admitted to the hospital to perform clinical and laboratoristic controls before the execution of cerebral axial tomography for the diagnosis of suspected isolated craniosynostosis.

Neurotrophic factor assays

Nerve growth factor (NGF): NGF levels were measured by a highly sensitive two-site immunoenzymatic assay which recognizes human and murine NGF and does not cross-react with BDNF. Polystyrene 96-well microtube immunoplates (Nunc) were coated with affinity purified, polyclonal goat anti-NGF antibody, diluted in 0.05 M carbonate buffer (pH 9.6). To evaluate the non-specific signal, parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA, USA). After overnight incubation at room temperature and 2 h incubation with a blocking buffer [0.05 M carbonate buffer (pH 9.5), 1% bovine serum albumine], the plates were washed three times with 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.5% gelatin, and 0.1% Triton X-100. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer [0.1% Triton X-100, 100 mM Tris-HCl (pH 7.2), 400 mM NaCl, 4 mM ethylenediamine-tetraacetic acid, 0.2 mM phenylmethylsulfonil fluoride, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% bovine serum albumine and 0.5% gelatin]; they were then distributed among the wells and left to stand overnight at room temperature. The plates were then washed three times and incubated with 4 mU/well anti-ß-NGF-galactosidase (Boehringer Mannheim, Germany) for 2 hours at 37°C; after further washing, 100 µl of substrate solution (4 mg/ml of chlorophenol red, Boehringer Mannheim, Germany, substrate buffer: 100 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 150 mM NaCl, 2mM MgCl2, 0.1% sodium azide and 1% bovine serum albumine) was added to each well. After incubation for 2 hours at 37°C, optical density was measured at 575 nm, using an ELISA reader (Dynatech), and the values of standards and samples were corrected by

taking non-specific binding into consideration. Under these conditions, the sensitivity was 3 pg/mL; the recovery of NGF in our assay ranged from 80 to 90%, and cross-reactivity with other molecules of the NGF family (i.e., NT-3 and NT-4/5) was less than 3%. NGF concentration was expressed as pg/mL for liquid samples. All assays were performed in triplicate.

Brain derived neurotrophic factor (BDNF): The endogenous BDNF was quantified using a two-site enzyme immunoassay kit (Promega, USA). As performed for the NGF assay, 96-well immunoplates (Nunc) were coated with 100 µl per well of monoclonal anti-mouse-BDNF antibody and incubated overnight at 4°C. The plates were then washed three times with wash buffer, and the samples were incubated, with shaking, in the coated wells (100 µl each) for 2 h at room temperature. After additional washes, the antigen was incubated, with shaking, with an anti-human BDNF antibody for 2 h at room temperature. The plates were washed again with wash buffer and then incubated with an anti-IgY human purified for 1 h at room temperature. After another washing, the plates were incubated with a tetramethylbenzidine/peroxidase substrate solution for 15 min, and 1M phosphoric acid was added to the wells (100 µl/well). The colorimetric reaction product was measured at 450 nm using an ELISA reader (Dynatech MR 5000, Germany). BDNF concentrations were determined from the regression line for the BDNF standard (ranging from 7.8 to 500 pg/ml-purified mouse BDNF), incubated under similar conditions in each assay. The sensitivity of the assay was 15 pg/mL of BDNF, and the cross-reactivity with other related neurotrophins (i.e., NGF, NT-3, and NT-4/5) was less than 3%. BDNF concentration was expressed as pg/mL for liquid samples. All assays were performed in triplicate.

Glial derived neurotrophic factor (GDNF): GDNF was quantified using a two-site enzyme immunoassay kit from Promega (USA) that differs only marginally from that used for BDNF. 96-well immunoplates (Nunc) were coated with 100 µl per well of monoclonal anti-GDNF antibody. After overnight incubation at 4°C, the antibody was removed from the plates and the samples were incubated, with shaking, in the coated wells (100 µl each) for 6 hrs at room temperature. The plates were then washed five times with buffer, and the antigen was incubated overnight with a polyclonal anti-human GDNF antibody at 4°C. The plates were washed again with buffer and incubated, with shaking, with an anti-chicken IgY conjugate for 2 h at room temperature. The remaining steps of the procedure were identical to those used for BDNF. The plates were incubated with a tetramethylbenzidine/peroxidase substrate solution for 15 min, and 1 M phosphoric acid (100 µl/well) was added to the wells. The colorimetric reaction product was measured at 450 nm. GDNF concentrations were also determined from the regression line for the GDNF standard (ranging from 15.6 to 1,000 pg/mL-purified human GDNF). The sensitivity of the assay was 30 pg/mL, and the cross-reactivity with other related neurotrophins (i.e., BDNF and NGF) was less than 5%. GDNF concentration was expressed as pg/mL for liquid samples. All assays were performed in triplicate.

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

Results

Clinical and laboratory differences between H1N1 patients and controls

We included in this study 15 patients with H1N1 virus infection, 15 children with LRTI, and 15 non-infected children defined "healthy children" (see Plasma sample collection). Patients with H1N1 infection were aged 1.1 years 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 (p=0.36), while 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 6 patients to the PIDU. Among children with LRTI, 8 out of 15 were admitted to the PICU with the diagnosis of severe RSV bronchiolitis, while the other 7 were admitted to the PIDU (4 with diagnosis of non-RSV bronchiolitis and 3 with diagnosis of influenza A (H2N3) virus infection). Regarding clinical differences between the two groups, H1N1 patients experienced higher median fever (39.2 °C) respect 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 respect to LRTI patients (median 6 days versus 4 days) (p<0.0001). The most frequent pulmonary abnormalities at chest x-ray were represented by pneumonia and pulmonary consolidation in the H1N1 patients, while in LRTI children we detected atypical findings, such as hyperinflated lungs and segmental pulmonary atelectasia. Two patients with H1N1 infection showed Pneumothorax (PNX), while another three children showed severe respiratory complications, such as pneumopericardium, pneumomediastinum, and pneumorrachis at chest CT scan (Table 1). No pulmonary or systemic complications were referred to LRTI group. No differences in clinical manifestations, such as gastrointestinal and neurological symptoms, have been reported between the two groups. Regarding laboratory tests (blood cells and platelet count, serum C-reactive protein, procalcitonin, GOT, GPT, CTN, 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, but H1N1 patients showed a significantly longer time of hospitalization respect to the control group (median 9 days versus 3 days: p=0.0013).

Neurotrophic factor expression in H1N1 patients and controls

In H1N1 patients we detected different plasma levels of Neurotrophic Factors. In these patients we found significantly (p<0.0001) higher levels of BDNF (326.3 ± 133.3 pg/mL) respect to NGF (31.2 ± 13.6 pg/mL) and GDNF (9.3 ± 4.1 pg/mL). Also in LRTI patients the mean plasma levels of BDNF were significantly higher respect 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) (Figure 1). No significant correlations were found between Neurotrophic Factor expression with the age of both patients and control group (p=0.35).

Plasma level differences of neurotrophic factor expression between H1N1 patients, LRTI controls, and "healthy children"

Significantly higher levels of BDNF and NGF were demonstrated in all patients with H1N1 infection respect to the controls, while GDNF levels did not undergo significant variations in the 2 groups.

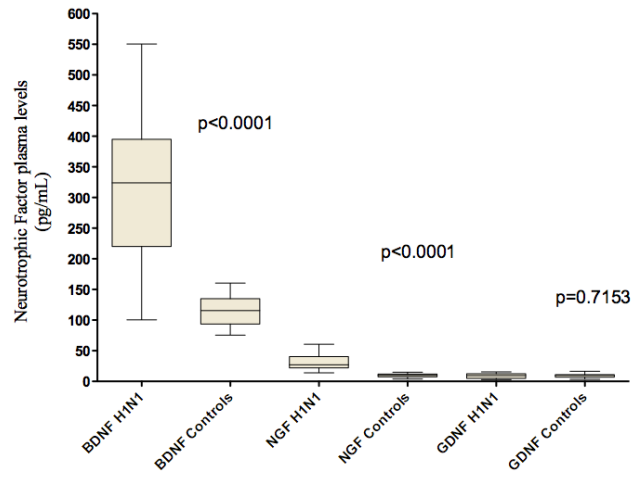

Figure 1: Box plot representation was used. H1N1 patients had significantly higher level of BDNF (p<0.0001) and NGF (p<0.0001) when compared to controls. There was no significant difference in GDNF level between the 2 groups (p=0.7153).

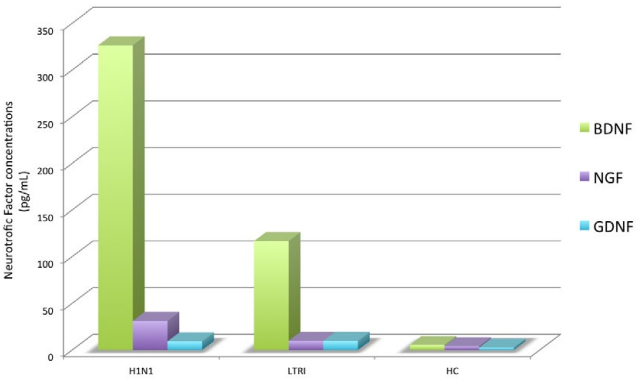

Figure 2: Levels of BDNF, NGF and GDNF are shown respectively in H1N1 patients (H1N1), LRTI controls (LRTI), and in "healthy children" (HC). Histogram representation was used.

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 we detected between the 2 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 1). No significant changes were observed in the GDNF plasma level between the 2 groups (9.3 ± 4.1 pg/mL in H1N1 patients compared to 9.8 ± 3.3 pg/mL in LRTI patients; p=0.71). Differently from H1N1 patients and LRTI controls, in "healthy children" we did not find significant differences between plasma levels of neurotrophins. In these children the mean plasma 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).

Correlation between neurotrophic factor expression with disease severity and clinical manifestations in H1N1 patients

To elucidate the association between neurotrophic factor expression and disease severity, we analyzed their plasma levels both in patients with severe (9 patients) and mild symptoms (6 patients) of H1N1 influenza virus infection. Compared to the mild patients, severe H1N1 patients produced significant higher levels of BDNF (404.6 ± 98.0 pg/mL versus 208.8 ± 82.6 pg/mL; p=0.0015), and NGF (38.4 ± 12.7 pg/mL versus 20.3 ± 4.8 pg/mL; p<0.0058), while no statistical differences we found in GDNF plasma levels between the two groups (8.1 ± 3.9 pg/mL versus 11.2 ± 3.8 pg/mL, p=0.152) (Figure 3).

Moreover, to verify whether there was a correlation between neurotrophic factor up-regulation and clinical manifestations in H1N1 patients we compared the plasma levels of these biomarkers with some clinical symptoms referred to the patients. Noteworthy, we detected a positive correlation between plasma level of NGF and duration of cough with a coefficient of determination of 0.37 (p=0.0165) (Figure 4). Moreover, in two patients with specific pulmonary complications, such as pneumothorax and pneumopericardium, we detected the highest NGF plasma levels of all H1N1 patients (60.5 pg/mL and 53.0 pg/mL, respectively). No significant correlations were reported between Neurotrophic Factor expression and other parameters evaluated, such as biochemical markers of inflammation (C-reactive protein and

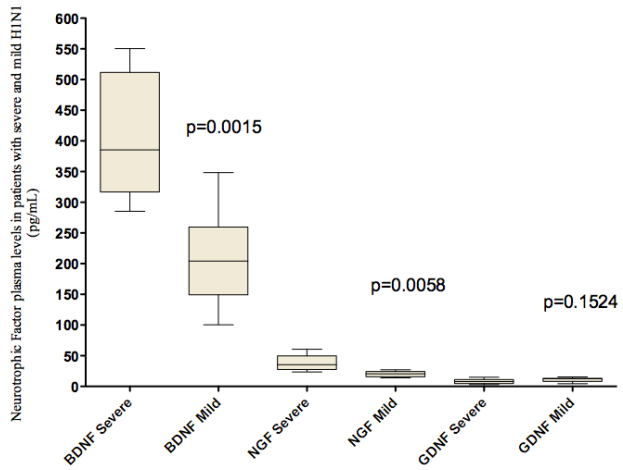

Figure 3: Box plot representation was used. H1N1 patients with severe disease had significantly higher level of BDNF (p=0.0015) and NGF (p=0.0058) when compared to patients with mild symptoms. There was no significant difference in GDNF level between the 2 groups (p=0.1524).

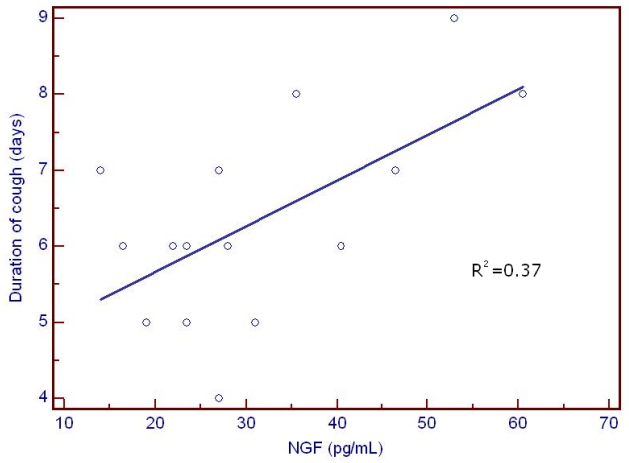

Figure 4: A scatter plot shows the relationship between the duration of cough and NGF plasma levels. The line represents the regression line (Linear regression equation: duration of cough = 0.06 x NGF + 4.5 R^2=0.37).

procalcitonin), systemic complications, and, finally, the outcome of children with H1N1 virus infection.

Discussion

Our study, despite the limited patient sample, provides evidence that H1N1 virus infection induces significantly increased plasma levels of 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 strongly BDNF and NGF up-regulation that correlates with the severity of clinical compromise assessed upon admission, while no differences were detected in GDNF expression in the two groups. We also observed that in H1N1 patients with more severe clinical manifestations of disease, plasma levels of both neurotrophins were significantly increased respect to H1N1 mild patients and that this up-regulation was correlated with some specific clinical manifestations and a longer time of hospitalisation. More in particular, NGF up-regulation was correlated with the duration of cough in this subset of patients. Neurotrophic factor up-regulation occurs soon after virus lung infection: it is early and differential and may be involved in modulating the response to virus infection, thus conditioning the outcome of the patients. Compared to LRTI controls and healthy children H1N1 patients showed a rapid and elevated plasma neurotrophic factor expression that appears markedly different among them, producing higher BDNF and NGF levels than GDNF. Our results are in keeping with other studies reported in literature showing that neurotrophin expression appears to be enhanced in different models of hypoxic-ischemic lung tissue, as induced by virus infection in H1N1 infected patients.

To date it is difficult to fully elucidate the role of neurotrophins in the mechanisms of 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 pro-inflammatory cytokines and neuropeptides [6]. Our results are in keeping with those findings showing 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. Noteworthy, we also observed that in H1N1 patients with more severe clinical manifestations of disease, plasma levels of both these neurotrophins were significantly increased respect to H1N1 mild patients and that this up-regulation was correlated with specific clinical manifestations, such as severity of pulmonary compromise and cough.

NGF, through its multiple actions on airway, immune and inflammatory cells, plays a key role in the bronchiolar hyperresponsiveness and cough [8,14,17]. Recent studies report a significant correlation between cough, asthma and increased NGF sputum levels suggesting a strong relationship between NGF up-regulation and severity of airway hyperresponsiveness [23,24]. 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 [6,13,18,25,26]. Our results are in keeping with those evidences because children with a longer cough and with specific pulmonary complications, such as pneumothorax and pneumopericardium, elicited higher NGF and BDNF plasma levels involved in the development of bronchial hyperreactivity, lung inflammation, and cough after H1N1 virus infection.

Up to now it is difficult to explain if the observed NGF and BDNF up-regulation in children with H1N1 virus infection can represent innate protective mechanisms for respiratory cell survival or it is secondary to a loss of physiological control on neurotrophin/neurotrophin receptor biosynthesis. It is possible that the biosynthesis of these neurotrophins becomes 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 [27,28]. Available clinical and experimental data does 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, were revealed to express BDNF and BDNF receptors in the experimental animal model of pulmonary sarcoidosis and chemical lung injury [29,30]. So, it is possible that neurotrophin up-regulation is secondary to lymphocytes rapid activation by H1N1 virus infection and that this over-expression represents an important process in the mechanisms of inflammatory host response after viral lung infections [18].

Previous studies, in fact, reported that different viral lung infections are associated with early neurotrophin biosynthesis, mainly BDNF and NT-3, suggesting that the changes of neurotrophin release may contribute to the development of lung inflammation and airway remodeling [18]. In our study NGF and BDNF up-regulation, observed early soon after H1N1 virus infection, was consistent with the timing of neurotrophin expression in experimental model of virus-infected human alveolar macrophages, suggesting that these neurotrophins act in different fashion to amplify and propagate inflammation in infected airways [31].

In conclusion, our observations provide a new evidence that a comprehensive neuroimmune response is activated at the early stage of pandemic H1N1 influenza virus infection with up-regulated production of NGF and BDNF. These findings are consistent with previous experimental and clinical studies confirming a key role exerted by neurotrophins in the pathogenesis of airway inflammation and hyperreactivity during virus lung infections. The increased expression of neurotrophins may be the underlying biochemical cause of the observed clinical symptoms in severe H1N1 patients and defining the relationships between 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.

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