

## Interleukin-8 is elevated in severe hand, foot, and mouth disease

Wen Wang<sup>1\*</sup>, Wei Li<sup>1\*</sup>, Xue Yang<sup>1</sup>, Tong Zhang<sup>1</sup>, Yuxuan Wang<sup>2</sup>, Rui Zhong<sup>1</sup>, Yanmei Jiao<sup>1</sup>, Tongzeng Li<sup>1</sup>, Taiyi Jiang<sup>1</sup>, Yakun Tian<sup>1</sup>, Hao Wu<sup>1</sup>

<sup>1</sup> Department of Infectious Diseases, Beijing You'an Hospital, Capital Medical University, Beijing, China

<sup>2</sup> Materials Science and Engineering Program, State University of New York at Binghamton, Binghamton, New York, United States

\* both authors contributed equally to this work

### Abstract

**Introduction:** Enterovirus 71 (EV71) infections can cause hand, foot, and mouth disease (HFMD), which is a potentially fatal illness in children. Epidemics of HFMD are seen every year globally and present an increasing threat to public health worldwide.

**Methodology:** To identify potential severity markers for severe HFMD, laboratory findings and levels of eight serum cytokines in 143 EV71-infected patients in Beijing You'an Hospital were analyzed. Patients were grouped by disease severity: Mild (no severe complications) (n = 59), isolated isolated brainstem encephalitis (BE) (n = 47), isolated pulmonary edema (PE) (n = 12), and BE+PE (n = 25).

**Results:** IL-8 levels peaked at day one after admission and were found to be correlated to disease severity, maximal body temperature, and length of hospital stay. Among all tested cytokines, IL-8 was correlated to only IL-6 (p = 0.010). IL-6 and IL-10 were elevated in most patients (98.6% and 70.6%), but not correlated to disease severity (both p > 0.05). IFN $\gamma$  was only negatively correlated to mild cases (p = 0.025).

**Conclusions:** IL-8 was correlated to disease severity of HFMD. IL-6 and IL-10, although elevated in most HFMD patients, were not correlated to disease severity.

**Key words:** interleukin-8; cytokine; hand, foot, and mouth disease; serum marker; disease severity

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### Introduction

Hand, foot, and mouth disease (HFMD), which was first reported in 1957, is a potentially life-threatening illness that is commonly seen in young children mostly younger than five years of age [1]. Enterovirus 71 (EV71), which belongs to the *Enterovirus* genus of the *Picornavirus* family, caused major HFMD outbreaks in recent years. Although present globally in most countries, the largest HFMD outbreaks have been seen in the Asia-Pacific area, especially in China, for unknown reasons [2-7]. HFMD is usually self-limited in mild cases, causing symptoms such as fever and vesicular exanthema on patients' soles, palms, and mouths. But the disease may progress to severe cases with fatal complications such as brainstem encephalitis (BE), pulmonary edema (PE), or both. Patients with BE or PE possess a remarkably higher mortality compared to patients with mild cases [6,8].

HFMD outbreaks increased in recent years, and severe cases were more commonly seen [9]. In this study, we sought to find markers that could predict the severity of the disease at the early stage of disease progression. Hyperglycemia and leukocytosis have been found to be two important markers, and have been used in clinical practice [10-12]; however, these two markers are not sensitive and specific enough for HFMD. Additional markers are required to effectively identify potential life-threatening cases and help clinicians take preventive measures.

Cytokines are key mediators in the inflammatory process during viral infections. It has been proven that massive proinflammatory cytokines play critical roles in the pathogenicity of EV71 infection [13-16]. It has been reported that interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-1R $\alpha$ , IL-6, IL-10, IL-13, interferon gamma (IFN $\gamma$ ), granulocyte colony-stimulating factor (G-CSF), and tumor necrosis factor alpha (TNF $\alpha$ ) in serum and cerebral spinal fluid (CSF) were elevated significantly in EV71-infected

patients [14,16-18]. Administration of intravenous immunoglobulin (IVIG), an important treatment for patients with BE and EV71-infected patients that contains natural antibodies against cytokines, was found to reduce levels of IL-6 and IL-8 at early stages of EV71 infection, and to possibly prevent disease progression to PE [19]. A study using a neonatal mouse model demonstrated that IL-6 may play a critical role in EV71 infection [20]. These studies all indicate the importance of cytokines in the pathogenesis of EV71 infection.

The objective of this study was to summarize the clinical and laboratory data of a group of pediatric HFMD patients admitted to Beijing You'an Hospital and to evaluate the correlation between cytokines and disease severity. This study aimed to identify potential early serum marker(s) of disease severity so that prophylactic measures can be taken to reduce mortality.

## Methodology

### *Case definition*

The case definition is described elsewhere [17]. Briefly, EV71 infection was defined as the isolation of the virus from at least one site (throat swab, blood, stool, cerebrospinal fluid (CSF), or other) with a negative bacterial culture. Patients with other co-infections were excluded from this study. BE was defined as a disease characterized by myoclonus, ataxia, nystagmus, oculomotor palsies, and bulbar palsy in various combinations, with or without the use of neuroimaging to confirm. PE was defined as respiratory distress with tachycardia, tachypnea, rales, with or without frothy sputum, and a positive chest radiograph that showed pulmonary infiltrates without cardiomegaly.

### *Study population*

Information about the study population, which consisted of 143 children who met the case definition described above, was collected retrospectively. The patients were consecutively admitted to Beijing You'an Hospital, Capital Medical University (Beijing, PR China) between May and July 2012. Patients were divided into four categories based on severity: (1) Mild: patients with HFMD without BE or PE; (2) BE: HFMD patients complicated by BE; (3) PE: HFMD patients complicated by PE; (4) BE+PE: HFMD patients complicated by BE and PE.

### *Data source*

All of the parameters, except kinetic level changes of IL-8, included in the investigation were collected by reviewing the patients' medical records, which were preserved in the medical record library and the medical computerized database at Beijing You'an Hospital, Capital Medical University. The patient records were retrospectively examined for the primary set of data, which included demographic characteristics (age and sex), clinical parameters (signs and symptoms), laboratory values (hematologic, biochemical, and microbiological findings), radiologic data, outcome at discharge (recovered or died), admission and discharge dates, and length of hospital stay. For kinetic level changes of IL-8, 12 patients (three cases of Mild, BE, PE and BE+PE each) were enrolled.

The Youan Hospital Ethics Committee approved this study. It covered the retrospective analysis of the 143 records and the additional study of cytokine levels in 12 patients. Parents or caretakers of all 143 participants and additional 12 patients for cytokine research gave written informed consent on behalf of the child participants for their information to be stored and used for research. Human experimentation guidelines of PR China were followed in the conduct of this clinical research.

### *Cytokine level determination*

The blood samples of all patients for cytokine determination were collected upon admission, and cytokine levels were tested by two trained technicians immediately. Among 143 selected cases, 12 typical patients were selected and blood samples were collected at day zero, day one, day two, day three, and day four after admission. The plasma was harvested within 30 minutes at 37°C of venipuncture from EDTA-anticoagulated blood samples and stored at -70°C until analyzed. The Bio-Plex Human 8-plex kit (Bio-Rad, Berkeley, USA) was used to detect IL-2, IL-4, IL-6, IL-8, IL-10, IFN $\gamma$ , GM-CSF and TNF $\alpha$  levels on Luminex200 xMAP analyzer system (Luminex, Austin, USA), according to the manufacturer's instructions. The cutoff values for IL-8 levels, to form a binary variable, were determined using the receiver operator characteristic curve to predict the severity of HFMD.

### *Statistical analysis*

The average of data was presented as median  $\pm$  interquartile range. Categorical data were tested using  $\chi^2$  or Fisher's exact test. Student's *t* test and the

Wilcoxon rank-sum test were used for analysis of continuous variables. Correlation was determined by Pearson correlation analysis, unless stated otherwise. The Mann-Whitney *U* test was used for data that did not have a normal distribution. All analyses were performed by SPSS software version 11.0. A *p* value < 0.05 was considered to be significant.

## Results

### *Patient characteristics and clinical and laboratory findings*

The data of all 143 hospitalized patients with HFMD in Beijing You'an Hospital between May and July 2012 were collected. The demographic and clinical data are summarized in Table 1. The average time for either fever or rash to appear after hospital admission was  $1.91 \pm 0.89$  days. Patients were subdivided into four groups – Mild, BE, PE, and BE+PE – as described above. Patients from Mild, BE, and PE groups all recovered and were discharged. One patient (0.7%, 1/143) in the BE+PE group died.

Age in Table 1 was analyzed by nonparametric ANOVA. No significant difference was found between groups (all *p* > 0.05). Among these patients, 88 (61.5%) were male, and 55 (38.5%) were female. The median age and the interquartile range of HFMD patients were 2.00 and 1.00 years of age, respectively. Skin rash and fever > 37.2°C were found in 100% of patients. A significant number of patients had a fever higher than 39°C upon admission, but analysis indicated no predictive value of fever on disease severity (having BE or/and PE or not, *p* > 0.05). Similar results applied to WBC count. Neutrophil and lymphocyte count elevations did not show significant difference among different groups of patients (*p* > 0.05). Among all 143 patients, no red blood cell (RBC) count, total bilirubin (TBIL), direct bilirubin (DBIL), albumin (ALB), total protein (TP), gamma-glutamyl transferase (GGT), prothrombin time (PT), blood urea nitrogen (BUN), or creatinine (Cr) abnormality was observed (data not shown).

### *Plasma cytokine levels of HFMD patients*

Cytokines are key mediators of different stages of immune responses to viral and bacterial invasion. Therefore, the levels of IL-2, IL-4, IL-6, IL-8, IL-10, IFN $\gamma$ , GM-CSF, and TNF $\alpha$  in all four groups of subjects were tested upon admission, and results are presented in Table 2.

Most tested cytokines were positively correlated to each other (all *p* < 0.05 by Spearman correlation analysis), except IL-8 and IFN $\gamma$ , which were not

related. There appears to be an activation of a well-connected cytokine network upon EV71 infection. The IL-10 levels were elevated in almost all patients (98.6%, 141/143, cutoff value = 1.53 pg/mL), followed by IL-6 levels (70.6%, 101/143, cutoff value = 8.53 pg/mL). IFN $\gamma$  was above normal range (124.08 pg/mL) in 42.0% (61/143) of patients. Although the levels of these three cytokines were significantly increased in HFMD patients compared to the normal population, there were no significant changes among patients with different disease severity (all *p* > 0.05, BE, PE, or BE+PE group vs. Mild group).

### *IL-8 is correlated to the severity of HFMD*

Among all tested cytokines, IL-8 (cutoff value = 116.07 pg/mL) was the only one found to be significantly higher in BE, PE, and BE+PE groups compared to the Mild group. IL-8 level in BE+PE group was the highest of all four groups (*p* < 0.01 vs. Mild, *p* < 0.05 vs. BE or PE group).

Among all tested cytokines, only elevated IL-8 was found to be significantly positively linked to BE+PE (*p* = 0.002) and negatively linked to mild cases (*p* = 0.039). In addition, elevated IFN $\gamma$  was negatively associated with mild cases (*p* = 0.025). These results strongly suggest the role of IL-8 in the pathogenesis of brainstem encephalitis and pulmonary edema in HFMD patients (Table 2). In addition, the levels of IL-8 were also positively correlated to maximal body temperature (*T*<sub>max</sub>, *p* = 0.036), and hospital days (*p* < 0.001) by Pearson analysis. The levels of IL-8 were negatively linked to lymphocytes and PLT (*p* = 0.048 and 0.022, respectively), but were not related to WBC (white blood cell count), neutrophil percentage, CK-MB, or liver function tests (all *p* > 0.05). Since cytokines are well connected to each other, the cytokine related to the elevation of the IL-8 level was sought. Among the selected cytokines, only IL-6 was positively related to IL-8 (*p* = 0.010).

To further investigate the effects of IL-8 elevation on the severity of HFMD, all 143 patients were divided into three groups, IL-8VH: very high level group, where the level of IL-8 was over 200 pg/mL; IL-8H: high level group, where IL-8 was between 120-200 pg/mL; IL-8L: low level group, where IL-8 was under 120 pg/mL, which was the upper limit of the normal range of serum IL-8. All patients (7/7, 100.0%) in the IL-8VH group were complicated with BE or PE, and 71.4% (5/7) with both. These ratios were much higher than in the IL-8H group (70.0% and 30.0%) and the IL-8L group (55.6% and 13.5%).

**Table 1.** Patient characteristics

	<b>Total (n = 143)</b>	<b>Mild (n = 59)</b>	<b>BE (n = 47)</b>	<b>PE (n = 12)</b>	<b>BE+PE (n = 25)</b>
Age (years)	2.45 ± 1.68	2.79 ± 1.96	2.25 ± 1.57	2.17 ± 1.1	2.38 ± 1.24
Gender (Male), %	88 (61.5%)	39 (66.1%)	30 (63.8%)	4 (33.3%)	15 (60.0%)
Fever > 39 °C	94 (65.7%)	39 (66.1%)	33 (70.2%)	7 (58.3%)	15 (60.0%)
WBC (x10 <sup>9</sup> /L)	11.9 ± 4.2	11.8 ± 4.6	11.9 ± 3.6	11.7 ± 4.86	12.1 ± 4.2
Neutrophils (%)	60.8 ± 16.2	61.1 ± 16.5	61.3 ± 13.8	57.2 ± 18.0	60.9 ± 19.5
Lymphocytes (%)	29.2 ± 17.3	27.6 ± 15.0	29.7 ± 15.0	41.0 ± 27.7	26.6 ± 19.1
HB (g/dL)	120.8 ± 18.5	120.8 ± 20.5	121.3 ± 21.3	123.1 ± 8.6	118.9 ± 9.6
PLT (x10 <sup>9</sup> /L)	291.2 ± 105.5	283.5 ± 109.7	306.7 ± 108.4	260.6 ± 56.1	295.2 ± 108.5
ALT (IU/L)	14.4 ± 10.1	14.9 ± 13.1	14.3 ± 9.1	13.4 ± 4.7	13.8 ± 4.1
AST (IU/L)	39.1 ± 19.8	40.1 ± 29.0	38.2 ± 10.5	37.4 ± 5.8	39.2 ± 7.0
ALB (g/dL)	44.7 ± 6.4	43.5 ± 9.1	45.8 ± 3.3	45.3 ± 2.9	45.5 ± 3.2
CK (IU/L)	106.2 ± 165.7	115.5 ± 214.3	96.0 ± 143.1	95.7 ± 63.5	108.6 ± 98.9
CK-MB (IU/L)	17.9 ± 12.7	18.0 ± 13.2	16.8 ± 13.6	23.4 ± 8.8	17.1 ± 11.0

BE: isolated brainstem encephalitis; PE: isolated pulmonary edema

**Table 2.** Plasma cytokine levels of HFMD patients

<b>Cytokine (pg/mL)</b>	<b>Total (n = 143)</b>	<b>Mild (n = 59)</b>	<b>BE (n = 47)</b>	<b>PE (n = 12)</b>	<b>BE+PE (n = 25)</b>
IL-2	24.8 ± 54.9	21.4 ± 49.2	31.4 ± 73.8	30.3 ± 41.7	18.0 ± 23.4
IL-4	2.3 ± 12.9	3.7 ± 20.1	1.3 ± 1.7	1.6 ± 1.4	1.1 ± 0.6
IL-6	48.5 ± 110.1	49.3 ± 95.8	44.0 ± 83.4	51.2 ± 65.6	42.4 ± 54.8
IL-8	53.1 ± 97.4	38.844.1±*	63.9 ± 109.0	39.4 ± 57.5	134.5 ± 198.8**
IL-10	36.4 ± 104.4	45.6 ± 125.8	41.1 ± 109.5	45.3 ± 84.6	13.9 ± 12.7
IFN $\gamma$	381.1 ± 1239.3	155.5 ± 143.1*	299.6 ± 463.5	306.8 ± 424.2	616.2 ± 1236.5
GM-CSF	44.2 ± 134.9	44.5 ± 74.7	39.0 ± 65.5	46.1 ± 53.8	48.2 ± 47.7
TNF $\alpha$	204.2 ± 915.1	133.3 ± 344.1	127.3 ± 309.1	934.6 ± 2783.9	77.7 ± 110.8

BE: isolated brainstem encephalitis; PE: isolated pulmonary edema; \* p&lt;0.05; \*\* p&lt;0.01

**Table 3.** IL-8 levels correlated to HFMD disease severity

	<b>Mild</b>	<b>BE</b>	<b>PE</b>	<b>BE+PE</b>	<b>Hospital days</b>
IL-8VH (n = 7)	0*	1 (14.3%)	1 (14.3%)	5 (71.4%)**	6.29 ± 1.113**
IL-8H (n = 10)	3 (30.0%)	4 (40.0%)	0	3 (30.0%)	5.70 ± 0.823*
IL-8L (n = 126)	56 (44.4%)*	42 (33.3%)	11 (8.7%)	17 (13.5%)	4.81 ± 1.282**

BE: isolated brainstem encephalitis; PE: isolated pulmonary edema; \* p&lt;0.05; \*\* p&lt;0.01



Statistical analysis indicated that IL-8VH was positively associated with BE+PE ( $p = 0.002$ ) and negatively with Mild ( $p = 0.041$ ), while IL-8L was positively associated with Mild ( $p = 0.039$ ) and negatively with BE+PE ( $p = 0.002$ ). Furthermore, hospital days were significantly and positively related to IL-8 levels ( $p < 0.000$ ,  $p = 0.029$  and  $0.006$  for IL-8L, IL-8H, and IL-8VH, respectively) based on the results of the Mann-Whitney test (Table 3). This data points to the conclusion that IL-8 level upon admission is correlated to disease severity of HFMD, and may be used to predict disease severity.

#### *Kinetic changes of IL-8 in patients with different severity*

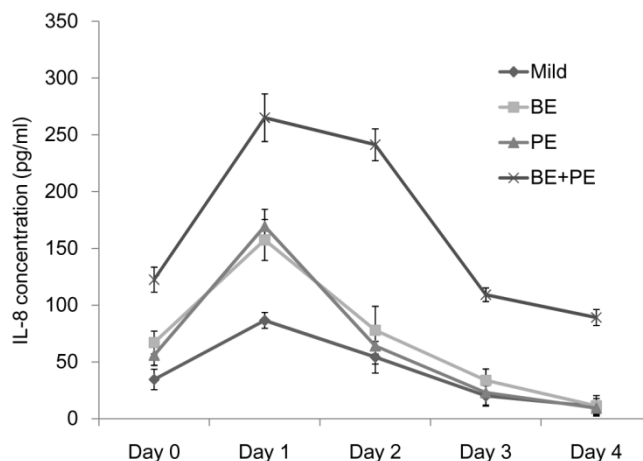
HFMD is a progressive disease, and the level of IL-8 changes along disease progression. Therefore, the kinetic changes of IL-8 levels in a small group of patients with different disease severity were monitored. In each group (Mild, BE, PE, and BE+PE) of HFMD patients, the IL-8 levels of three typical patients were tested daily. Dynamic changes of IL-8 level are shown in Figure 1. In all four groups, the IL-8 levels peaked at day one after admission and started to decline thereafter. The IL-8 level in patients with BE+PE was constantly higher than the other three types of patients, and stayed high at day four, when that of the other three types of patients were back to low levels. These results confirm that IL-8 level is correlated to the severity of HFMD along disease progression.

#### **Discussion**

Interleukin-8 (IL-8), also named CXCL8, is a chemokine secreted by macrophages and other cell types such as epithelial and endothelial cells. It is responsible for the recruitment of inflammatory cells such as neutrophils [21]. The CSF IL-8 level is elevated in a broad range of CSF inflammatory disorders, such as encephalitis, traumatic brain injury, and CSF hemorrhagic disorders [22-24]. Recently, more and more studies show that IL-8 is involved in the pathogenesis of lung cancer [25]. There are also reports implicating IL-8 in the development of schizophrenia, a psychiatric illness [26]. Our findings showed that IL-8 is strongly related to the severity of HFMD, and may be used as a predictive marker in the early stages of the disease.

In our study, the level of IL-8 at admission was found to be correlated to disease severity. Patients with BE or PE had higher IL-8 levels (both  $p < 0.05$ ), and maximal IL-8 levels were seen in the BE+PE

**Figure 1.** Kinetic changes of IL-8 in HFMD patients with different severity



Y axis: IL-8 concentration (pg/mL); X axis: Time points after admission when IL-8 levels were tested. Mild: HFMD patients without severe complications; BE: HFMD patients complicated with brainstem encephalitis; PE: HFMD patients complicated with pulmonary edema; BE+PE: HFMD patients with both BE and PE

group (Table 2). This association was further clarified by stratifying all patients by IL-8 level upon admission. In the IL-8VH group, all patients were complicated with BE or PE, and most cases had BE+PE, while lower percentages were found in IL-8H and IL-8L groups (Table 3). In addition, we found that IL-8 level was associated with maximal body temperature ( $p = 0.036$ ) and length of hospital stay ( $p < 0.001$ ). Data on the kinetic observation of IL-8 level since admission of HFMD patients with different severity demonstrated that IL-8 level peaked at day one after admission, and in the BE+PE group, IL-8 levels were much higher and lasted longer than normal compared to other groups with milder cases (Figure 1). All results indicated a strong correlation between IL-8 and disease severity. As a proinflammatory chemokine, IL-8 has been reported to be involved in several diseases of respiratory and neurological disorders. Its important regulatory roles have been found in many systems tested *in vitro* and *in vivo* [25-27]. It is reasonable, therefore, to see elevated IL-8 in HFMD with BE and PE. Most of our tests were done on patients upon admission, when the disease was at its early stage ( $1.91 \pm 0.89$  days on average from symptom appearance to admission). Therefore, our data suggest that patients with high levels of IL-8 at admission may develop severe complications such as BE, PE, or both. The specific roles of IL8 and the mechanisms on how it is involved require further study.

Because most cytokines are involved in signaling networks and may cross-regulate each other, we tested the correlation among selected cytokines. Among all tested cytokines, IL-8 was only correlated with IL-6 ( $p < 0.05$ ). TNF $\alpha$  was known to be able to upregulate IL-8 in keratinocytes [28]. However, in our data, they were not correlated ( $p > 0.05$ ).

IL-1 $\beta$ , IL-1R $\alpha$ , and G-CSF were recently reported by Griffiths *et al.* as prognostic markers for EV71-infected hospitalized Malaysian children [18]. They tested IL-8 as well but did not find any significant changes. This may be due to different technologies used for cytokine level detection, or to racial difference. Here, our results strongly support that IL-8 is correlated with the disease severity of HFMD.

In conclusion, our results show that IL-8 is strongly associated with the disease severity of HFMD, and may be used as a predictive marker for disease progression. IL-6 and IL-10, although elevated in patients with HFMD, are not related to disease severity.

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### Corresponding author

Hao Wu  
Department of Infectious Diseases, Beijing You'an Hospital,  
Capital Medical University  
Beijing 100069, China  
Phone: 086-010-83997162  
Fax: 086-010-83997164  
Email: wuhao90000@gmail.com

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