

# Determination and clinical correlation of markers of inflammation in unvaccinated patients with varicella-zoster infection

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**Abstract.** – **BACKGROUND:** Chicken pox is commonly known as a benign exanthematous disease of childhood, occasionally neurologic or hemorrhagic complications, or even death may ensue. Early predictors of severity of disease have yet to be identified. TNF-alpha and IL-6 stimulate virus-specific immunoglobulin production and it has been postulated that determination of levels of these cytokines may be useful as a prognostic factor.

**PATIENTS AND METHODS:** Patients who were diagnosed with a varicella-zoster virus (VZV) infection in the Outpatient Clinic of the Department of Pediatric Infectious Diseases were evaluated for eligibility. Laboratory assays included an evaluation of complete blood counts, erythrocyte-sedimentation rate (ESR), c reactive protein (CRP), and the number of tumor necrosis factor-alpha/interleukin-6-(TNF-alpha/IL-6-) producing mononuclear cells as determined by flow cytometry.

**RESULTS:** A total of 339 patients (320 with chickenpox and 19 with shingles) were enrolled. Blood samples could only be obtained from 81 of the 320 patients with chickenpox. Patients were also divided into three groups depending on the number of skin (vesicular) lesions. (group 1,  $\leq 50$  lesions; group 2, 51-100 lesions; group 3,  $>100$  lesions). Correlation analyses did not reveal the presence of a statistically significant correlation between number of skin lesions with either of white blood cells (WBC) count ( $p = 0.231$ ), ESR ( $p = 0.879$ ) or CRP ( $p = 0.373$ ). The mean percentage of TNF-alpha-producing mononuclear cells was significantly higher in group 2 compared to group 3 ( $p = 0.003$ ). A similar difference was observed with regard to IL-6-producing mononuclear cells, albeit bordering on statistical significance ( $p = 0.058$ ).

**CONCLUSIONS:** Decreased expression of the cytokines TNF-alpha and IL-6 may be responsible for the development of a more severe clinical picture in patients with VZV infection, and determination of intracellular levels of these cytokines may be of benefit for early identification of patients who may have a more severe clinical course.

*Key Words:*

Children, Varicella, Zoster, Acute phase reactants, IL-6, TNF-alpha.

## Introduction

Varicella, caused by the varicella zoster virus (VZV), is one of the most common infectious diseases of childhood in unvaccinated community. VZV is known to result in varicella (chickenpox) and herpes zoster (shingles)<sup>1,2</sup>. Varicella is considered the primary infection of the virus, which is an infectious disorder of childhood characterized by fever and a widespread vesicular, itchy rash<sup>2,3</sup>.

Herpes zoster is a latent infection which develops as a result of reactivation of virus particles which remain dormant in the dorsal ganglion after an initial varicella infection. Herpes zoster usually presents at a more advanced age, typically as vesicular lesions on the dermatome innervated by the affected ganglion<sup>2,3</sup>.

Although most cases of varicella are self-limited, that may be followed-up at an outpatient basis, serious complications which require hospitalization may occur. In a study spanning an 8 year period prior to licensing of the varicella vaccine, an estimated 10632 annual hospitalizations were attributable to varicella infection<sup>4</sup>, with children aged  $< 5$  years accounting for up to 44% of hospitalizations, while 33% of individuals were between the age of 5-20 years<sup>4,5</sup>. Ages of  $< 12$  months or  $> 20$  years were associated with 6-fold and 13-fold increases in risk of hospitalization, respectively<sup>4</sup>. The most commonly encountered complications that required hospitalization were skin and soft tissue infections, pneumonia, dehydration, and encephalitis<sup>4</sup>. Varicella-related deaths have also been reported in the literature.

Taking into consideration the significant morbidity and mortality associated with varicella infections in children, predictors of a more severe course of disease would help clinicians to identify high-risk patients. Since routine use of antiviral treatment is not recommended for varicella infections, such markers would help arrive at a decision for early initiation of anti-viral treatments which would in turn result in better clinical outcomes.

The aim of this study was to establish factors that may be used in the early period of an infection as an indicator of poor prognosis in patients with varicella infections, as well as to determine their value as reliable markers in clinical practice. The other goal of the study was to determine any differences between primary varicella infections and herpes zoster in terms of demographic characteristics, routine laboratory tests, acute phase reactants and immune responses.

## Patients and Methods

### *Patient Selection*

This prospective study was undertaken in the Outpatient Clinic of the Department of Pediatric Infectious Diseases at Hacettepe University Faculty of Medicine with the approval of the local Ethics Committee. Children (6 months-18 years) diagnosed with chickenpox or herpes zoster were considered candidates for inclusion in the study. A diagnosis of chickenpox was made in the presence of a polymorphic vesicular rash in patients with history of exposure to other infected individuals.

Patients with characteristic vesicular lesions on a specific dermatome, with or without neuralgia, who also gave a history of a previous varicella infection, were considered to have herpes zoster.

For the purpose of this study, a clinical diagnosis was deemed sufficient for VZV infections, and no further laboratory test were performed to confirm a diagnosis. The parents/legal guardians of patients with a confirmed diagnosis were approached regarding the possibility of enrolling their children in the study, and only the children of consenting parents/guardians were screened for eligibility. Exclusion criteria included a confirmed history or a suspicion of a congenital immune disorder, any chronic illness, an acute infection within the previous month, trauma and/or fracture within the previous 6 months, suspicion

of another source of infection particularly bacterial in origin, current use of steroids (including inhalers), nonsteroidal anti-inflammatory drugs or antihistamines, a duration of symptoms earlier than 48 hours and more than 72 hours.

### *Blood Sampling and Laboratory Assays*

Venous blood samples were obtained within 2 hours of confirmation of a diagnosis of a VZV infection. For each patient, approximately 2.5 ml of blood was transferred into Na-heparin tubes for the evaluation of TNF- $\alpha$  and IL-6 as well as for intracytoplasmic staining, while the remainder of the obtained blood was sent for routine testing. The tests that were performed included a complete blood count on an automated "Counter CBC" hemocytometer, determination of erythrocyte sedimentation rate (ESR) by the Westergren method, and measurement of C-reactive protein (CRP) levels by the nephelometric method on an IMAGE 800 immunochemistry device (Beckman Coulter Inc., Galway, Ireland) using original reagents.

### *Determination of Intracellular TNF- $\alpha$ and IL-6 Levels in Peripheral Blood Mononuclear Cells<sup>6</sup>*

Separation of mononuclear cells from peripheral blood is performed by first diluting a blood sample with phosphate buffered saline (PBS) at a ratio of 1:1, followed by separation of mononuclear cells using the Ficoll gradient method. After centrifugation of the mixture for 30 minutes at 400  $\times$  g on a Pharmacia Biotech<sup>R</sup> device (Uppsala, Sweden), the resulting supernatant is extracted and distributed equally into tubes (approximately 2  $\times$  10<sup>6</sup> cells per tube) which are then each combined with 100  $\mu$ L of cell culture medium.

The measurement of cytokine levels in the endoplasmic reticulum and the Golgi apparatus requires cells to be in a state of stimulation, which is achieved by adding 50 ng/ml of PMA (Sigma<sup>R</sup>, St Louis, MO, USA catalog no: P.8139) and 500 ng/ml of ionomycin (Sigma<sup>R</sup>, catalog no: 1.0634, USA).

To facilitate the accumulation of sufficient amounts of intracellular cytokines to allow for enhancement of the signaling pathways of fluorescent cytokines, 3  $\mu$ L of 1 mM monensin (GolgiStop<sup>R</sup>, Becton-Dickinson, Pharmingen, San Diego, CA, USA), a carboxylic ionophore protein transport inhibitor are added to the mixture. Samples are then incubated in 5% CO<sub>2</sub> at a tem-

perature of 37°C for 4 hours, after which cells were washed with 3 ml of PBS. This is followed by centrifugation for 5 minutes at a temperature of 4°C with a speed of 250 × g. The resulting supernatant is extracted.

Prior to staining of intracellular cytokines with anti-cytokine antibodies, fixation and increased permeability of cells is achieved by adding 250 µL of Cytofix/Cytoperm<sup>R</sup> (Becton-Dickinson, Pharmingen, San Diego, CA, USA), a solution containing paraformaldehyde and saponin. Tubes are then stored in a dark room at 4°C for 20 minutes.

Cells are then washed by adding them to 100 µL of PermWash<sup>R</sup> solution (Becton-Dickinson, Pharmingen, San Diego, CA, USA) for a period of 15 minutes, followed by centrifugation for 5 minutes at 250 × g. The resulting supernatant is extracted and the same washing process is repeated once more.

To prevent non-specific background immunofluorescent staining, Fc receptors are blocked by adding 0.5 ml 20 µg/ml murine gammaglobulin at freezing temperatures. The now fixated cells with increased permeability are then washed in a solution containing a mixture of 100 µL PermWash<sup>R</sup> solution, anti-human-TNF-alpha and anti-human IL-6 phycoerythrin (PE)-bound<sup>7</sup> (Becton-Dickinson<sup>R</sup>, Pharmingen, San Diego, CA, USA) anti-cytokine antibody. FITC- and PE-bound antibodies provide different fluorescent colors. Cells are then incubated for 30 minutes in a dark room at 4°C after which they are washed with PermWash<sup>R</sup> solution followed by centrifugation for 5 minutes at 250 × g and extraction of the resulting supernatant. The washing process is repeated twice more before addition of 0.5 ml of a buffered fixative. The mixture is then stored in a dark room at 4°C until flow cytometric analysis is performed.

### **Flow Cytometric Analysis**

Three-color flow cytometric analysis was performed on an EPICS XL flow cytometer with System II, version 1.0, software (Coulter, Miami, FL, USA) that had been calibrated daily with DNA-Check-Beads (Coulter, Miami, FL, USA). Data were expressed as the percentage of cytokine-producing lymphocytes and monocytes.

### **Statistical Analysis**

All statistical analyses were performed using the SPSS package program for Windows®, version 15.0 (SPSS Inc., Chicago, IL, USA). Com-

parisons were made between patients with herpes zoster and primary varicella infection. Categorical parameters, such as gender, were compared using the Chi-square test. Before testing the difference between groups, parametric test assumptions were controlled. Normality of the continuous variables was tested by Shapiro-Wilk test. Homogeneity of variance was determined by Levene test. For continuous variables, either the Student's *t*-test or the Mann-Whitney U test were used depending on parametric test assumptions. Patients were also divided into three groups depending on the number of skin (vesicular) lesions. (group 1, ≤ 50 lesions; group 2, 51-100 lesions; group 3, >100 lesions). Association between number of lesions and gender were evaluated using the Chi-square test. Difference between the number of skin lesion groups were determined by one way analysis of variance or Welch analysis of variance according to parametric test assumptions were provided. Post hoc comparisons were done by Tukey HSD or Games-Howell test. In the event of a nonnormal distribution, the Kruskal Wallis test was used. A *p*-value of < 0.05 was considered indicative of statistical significance.

## **Results**

A total of 339 patients were diagnosed with a VZV infection during the study period, 320 of which had varicella and 19 had herpes zoster. Of the 320 patients with primary varicella infection, 183 were male (57.2%) and 137 were female (42.8%) with a female-to male ratio of 0.75. The mean age of patients in the chickenpox group was 5.5±3.4 years (median 5; range 6 months-17 years). In terms of age distribution, 41 patients (12.8%) were less than 12 months old at the time of diagnosis, 31 (9.7%) between 13-24 months old, 32 (10%) between 25-36 months, 29 (9.1%) between 37-48 months, 32 (10%) were aged 5 years, 39 (12.2%) 6 years, 37 (11.6%) 7 years, 28 (8.8%) 8 years and the remaining 51 patients (15.9%) were aged 9 years or older.

The mean duration of skin lesions prior to presentation in patients with chickenpox was 69.6 hours. Only 60 of the children (18.75%) were brought to medical attention within 24 hours of the onset of symptoms. Of the 41 patients younger than 1 year of age, 95.1% were brought to our Outpatient Clinic within the first day of symptoms.

Four of the patients with chickenpox (1.25%) developed varicella pneumonia which required hospitalization and mechanical ventilation. All four patients were discharged.

The 19 patients diagnosed with herpes zoster had a mean age of 10.89±3.87 years (range 2-16 years). Being a requirement for a diagnosis of shingles, all patients had a history of previous chickenpox infections, 6 (31.57%) of which occurred within the first year of life and 5 (26.31%) between 1-2 years of age. The female-to-male ratio of herpes zoster patients was 0.9 (10 males and 9 females).

Blood samples were obtained from only 81 of 320 patients who presented to the Outpatient Clinic with chickenpox in 48 to 72 hours, 49 (60.5%) of which were male and 32 (39.5%) were female, with an overall mean age of 6.07 ± 3.65 years (range 6 months-16 years). All patients with herpes zoster had blood samples obtained.

A comparison of demographic characteristics of herpes zoster and varicella patients did not reveal a significant difference with regard to gender distribution, although patients with herpes zoster were significantly older as expected ( $p < 0.001$ ).

In terms of laboratory results, patients in the herpes zoster group had significantly higher values for mean hemoglobin ( $p = 0.04$ ) and percentage of TNF- $\alpha$ -producing mononuclear cells ( $p = 0.028$ ) as well as lower values for CRP ( $p = 0.01$ ) and ESR ( $p = 0.04$ ) compared to patients with varicella. The differences between groups regarding white blood cell (WBC) counts and percentage of IL-6-producing mononuclear cells were statistically insignificant (Table I).

In terms of the number of skin lesions, 25 (30.9%) patients were in group 1 ( $\leq 50$  lesions), 33 (40.7%) patients in group 2 (51-100 lesions) and 23 (28.4%) patients in group 3 ( $> 100$  le-

sions). The distribution of patients according to age and number of skin lesions is summarized in Figure 1 and flow chart of the study seen in Figure 2. One of the patient who required hospitalization was in group 2 and three of them were in group 3.

Results of levels of acute phase reactants, as well as the percentages of cytokine-producing mononuclear cells for all three groups have also been summarized in Table II. Statistical analyses did not reveal the presence of a statistically significant relation between number of skin lesions and either of WBC count ( $p = 0.231$ ), ESR ( $p = 0.879$ ) or CRP ( $p = 0.373$ ). The mean percentage of TNF- $\alpha$ -producing mononuclear cells was significantly higher in group 2 compared to group 3 ( $p = 0.003$ ). A similar difference was observed with regard to IL-6-producing mononuclear cells, albeit bordering on statistical significance ( $p = 0.058$ ). These findings have been summarized in Figure 3 and Figure 4.

## Discussion

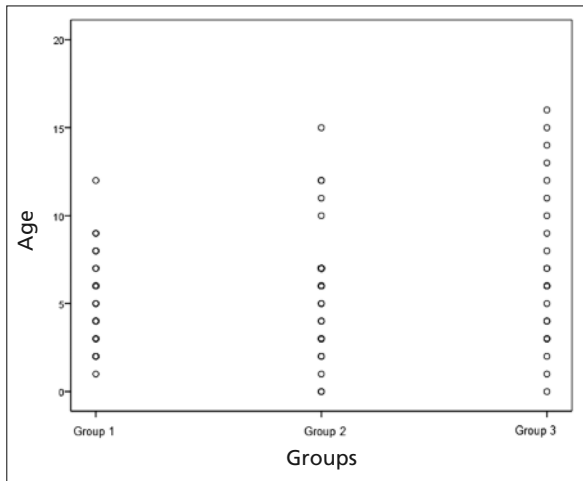
Varicella is highly infectious, with reported attack rates in susceptible contacts ranging from 61% to 100%<sup>10</sup>, the highest incidence occurring in children aged between 1-9 years<sup>11-14</sup>). Recent studies have shown an increase in the number of cases of varicella infections among very young children, particularly under the age of 5 years<sup>11,15,16</sup>).

Nearly three-quarters of patients who present to our Outpatient Clinic are of preschool age (7 years), which facilitates the spread of this highly contagious infection as children are introduced into the crowded conditions of nurseries and day-care centers. The increased prevalence of varicella in patients under the age of 2 years could be related to better parent awareness resulting in early visits to the doctor within 24 hours of the

**Table I.** Comparison of Hb, WBC, ESR, CRP, TNF- $\alpha$  and IL-6 in patients with varicella and herpes zoster groups.

Parameter	Varicella (n = 81)	Herpes zoster (n = 19)	p
Hb (gr/dL)	12.05 ± 1.13	12.92 ± 1.21	0.004
WBC (/mm <sup>3</sup> )	6532 ± 2242	7431 ± 2351	> 0.05
ESR (mm/h)	20 [6-60]	12 [3-35]	0.004
CRP (mg/dL)	0.78 [0.09-12.62]	0.34 [0.10-1.40]	0.001
TNF- $\alpha$ (%)	3.38 ± 2.11	4.67 ± 2.81	0.028
IL-6 (%)	3.12 ± 1.64	3.06 ± 1.87	> 0.05

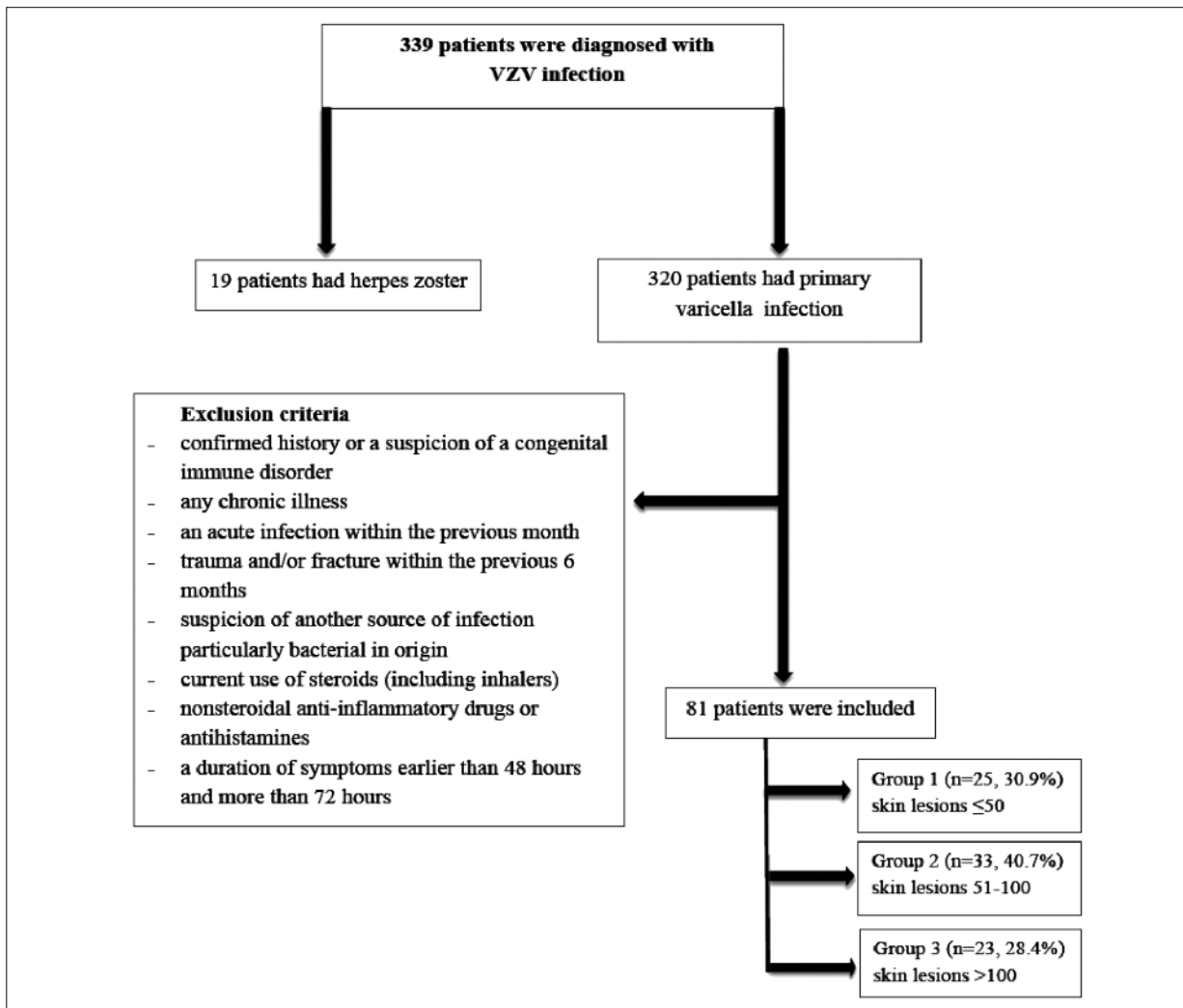
WBC: white blood cell count; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; TNF- $\alpha$ : tumor necrosis factor alpha; IL-6: interleukin 6. Variables showed Mean ± SD or median [min-max].



**Figure 1.** Distribution of patients according to age and number of skin lesions.

onset of symptoms. This is supported by our finding that 95% of patients under the age of 12 months were brought to medical attention within 24 hours, compared to the overall mean time to presentation of 69.6 hours when patients of all ages are considered.

Hospital admission rates for varicella infections range from 2.2 to 4.7 per 1000 cases in several national studies from the United States (USA), United Kingdom (UK) and France<sup>4,7-9</sup>. The annual frequency of varicella infections evaluated on an outpatient basis in the Department of Pediatric Infectious Diseases at Hacettepe University, which has a patient load of 2500 patients per year, is 5.6 per 1000 cases. When the number of cases that also present to the Emergency Department are taken into consideration, it may be deduced that varicella is a more important public



**Figure 2.** Study design and flow chart.

**Table II.** Values of various laboratory parameters according to the number of skin lesions.

Parameter	Group 1 (n = 25)	Group 2 (n = 33)	Group 3 (n = 23)	p
Hb (gr/dL)	12.35 ± 0.87	11.95 ± 1.05	11.89 ± 1.45	> 0.05 <sup>a,b,c</sup>
WBC (/mm <sup>3</sup> )	6600 [4300-12000]	5900 [3600-13200]	5500 [3400-13700]	> 0.05 <sup>a,b,c</sup>
ESR (mm/h)	20 [6-42]	20 [10-51]	22 [6-60]	> 0.05 <sup>a,b,c</sup>
CRP (mg/dL)	0.69 [0.10-6.72]	0.68 [0.24-9.33]	0.93 [0.09-12.62]	> 0.05 <sup>a,b,c</sup>
TNF-α (%)	3.09 ± 1.75	4.27 ± 2.31	2.42 ± 1.69	0.073 <sup>a</sup> > 0.05 <sup>b</sup> 0.003 <sup>c</sup>
IL-6 (%)	2.84 ± 1.52	3.66 ± 1.86	2.65 ± 1.23	> 0.05 <sup>a,b</sup> 0.058 <sup>c</sup>

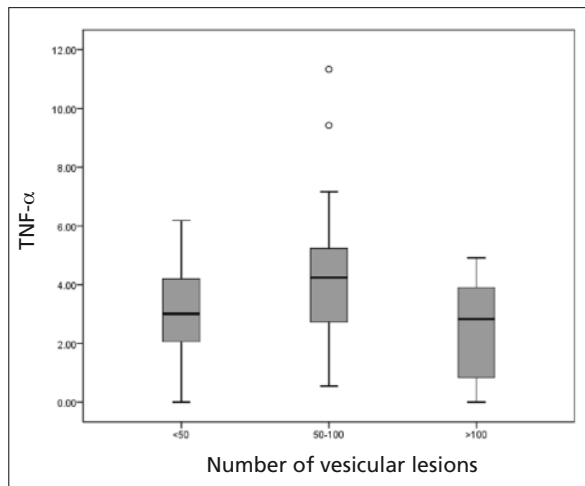
<sup>a</sup>Group 1 versus Group 2, <sup>b</sup>Group 1 versus Group 3, <sup>c</sup>Group 2 versus Group 3; WBC, white blood cell count; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; Variables showed Mean ± SD or median [min-max].

health problem in Turkey, when compared to Europe and the USA.

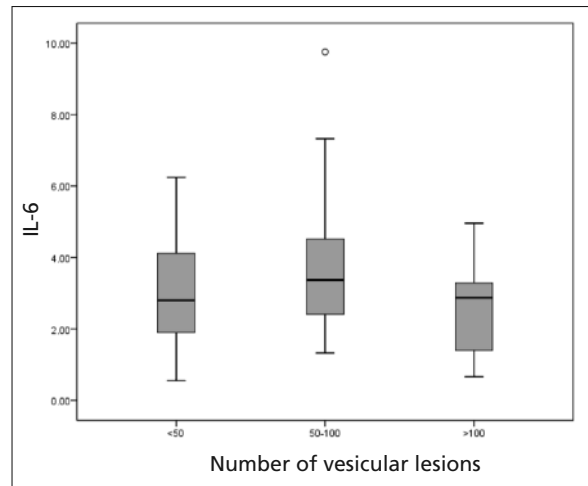
The most common complications associated with varicella that required hospitalization are secondary skin and soft tissue infections, pneumonia, dehydration, and encephalitis<sup>4</sup>. Prior to the introduction of the varicella vaccine into the routine vaccination program of the USA, an estimated 10,632 hospitalizations were attributable to varicella annually (rate: 2.3-6.3 per 100,000 population)<sup>4</sup>. In our study 1.25% of patients with varicella were hospitalized due to pneumonia. Early diagnosis and prompt initiation of effective

supportive treatment, including mechanical ventilatory support, were probably responsible for the favorable outcomes observed in our patients, since all four were eventually discharged.

The seemingly high hospitalization rate observed in our investigation could be related to the fact that our Hospital is a tertiary center to which more severe and complicated cases are usually referred to. It is widely believed that incorporation of the varicella vaccine into the national childhood immunization schedule would result in a significant reduction in Hospital visits and admissions related to VZV infections.



**Figure 3.** Box-and-whisker plots depicting the variations in TNF-α levels according to the number of skin lesions (<50, 51-100, >100). Median values are represented by the solid black lines, while the ends of the boxes represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles. The whiskers indicate the range of TNF-α levels for each group. Open circles and asterisks represent outlier observations according to the graphic box plot criterion (points that are beyond the quartiles by one and a half and three times the interquartile range, respectively).



**Figure 4.** Box-and-whisker plots depicting the variations in IL-6 levels according to the number of skin lesions (<50, 51-100, >100). Median values are represented by the solid black lines, while the ends of the boxes represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles. The whiskers indicate the range of IL-6 levels for each group. Open circles and asterisks represent outlier observations according to the graphic box plot criterion (points that are beyond the quartiles by one and a half and three times the interquartile range, respectively).

Herpes zoster (shingles) develops as a result of reactivation of latent VZV. Advanced age and the presence of an underlying immune deficiency are well known risk factors for the development of herpes zoster<sup>17</sup>. As would be expected, our patients with herpes zoster infection were significantly older than patients with varicella. Polymorphisms in the genes coding for interleukin-10 and other specific cytokines have been implicated as a possible cause of an increased general susceptibility to infections as well as for the reactivation of VZV, by affecting cell-mediated immunity<sup>17</sup>. In our caseload, patients with herpes zoster did not have significantly higher values for acute phase reactants. However, patients in the herpes zoster group had significantly higher values for mean hemoglobin ( $p = 0.04$ ) and percentage of TNF- $\alpha$ -producing mononuclear cells ( $p = 0.028$ ) as well as lower values for CRP ( $p = 0.01$ ) and ESR ( $p = 0.04$ ) compared to patients with varicella. The differences between groups regarding white blood cell counts and percentage of IL-6-producing mononuclear cells were statistically insignificant. The observed statistically significant differences could be attributed to the fact that compared to herpes zoster, varicella is a more widespread systemic infection.

The VZV virus has been shown to have "tropism" to T-cells of the immune system. Activation of CD4 and CD8 T-lymphocytes promotes the development of several immune mechanisms against the virus, which generally manifest 3 days after initial entry of the microorganism into the host. The earliest host response involves lysis of fibroblasts and other virus-infected cells by natural killer (NK) cells. This cellular response is stimulated and maintained by several proinflammatory cytokines such as IL-2, IL-6 and TNF- $\alpha$ <sup>18-20</sup>.

With this study, our aim was to establish a link between intracellular levels of proinflammatory cytokines and disease severity in an attempt to evaluate the value of measuring cytokines as an early predictor of a more severe course of disease, an area which has not been extensively explored before. Our findings show that patients with between 51-100 skin lesions had a significantly higher ratio of peripheral mononuclear cells with elevated intracellular levels of TNF- $\alpha$  compared to patients with more than 100 skin lesions. Although a similar difference was observed with IL-6, this was bordering on statistical significance. Previous studies have demonstrated that the use of non-steroidal anti-inflammatory

drugs (NSADs) is associated with a more severe disease course and an increase in the frequency of severe skin infections<sup>21-23</sup>.

## Conclusions

From these studies, it may be speculated that a suppressed cytokine response may result in a more severe presentation. However, we could not demonstrate a direct correlation between a more severe clinical picture and low serum levels of TNF- $\alpha$  and IL-6. The relationship between disease severity and cytokine levels in patients with varicella infection needs to be evaluated on a larger scale on more subjects, using also modern methods for measuring serum cytokine levels. Further studies on immunocompromised and immunosuppressed patients would help elucidate the mechanisms behind this hypothesis.

## Conflict of Interest

None to declare.

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