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Dinamic changes of pro-inflammatory cytokines, adhesion molecules and lymphocytes activation markers as early indicators of diseases severity in patients with Dengue

(Cambios dinámicos de citoquinas proinflamatorias, moléculas de adhesión y marcadores de activación linfocítica como indicadores tempranos de severidad en pacientes con Dengue)

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Abstract (english)

Several immunopathogenic mechanisms have been proposed to explain the massive increase of vascular permeability observed in the severe forms of infection by Dengue Virus (DENV). Our aim was to determine the kinetic changes of inflammatory mediators (IL-8, TNF- α), soluble early lymphocyte activation markers (sIL-2R, sTNF-Rp75) and soluble fractions of cell adhesion molecules (sICAM-1 and sVCAM-1) as indicators for early recognition of disease severity in patients with laboratory-confirmed dengue. Twenty patients classified as Dengue±Warning Signs (D±WS) and thirty patients with Severe Dengue (SD) were included in the study. Serums of apparently healthy individuals were included as controls. Compared with normal subjects, D±WS cases did not show significant differences in the levels of IL-8 or TNF-α during the acute nor in the critical stages of the disease; however, in D±WS cases levels of sICAM-1 and sVCAM-1 were higher than controls during both phases; in contrast, significant increase of sTNF-p75 and sIL2R levels were observed during the critical phase of the disease. Compared with both dengue patients and controls, patients with SD showed significant rise in the levels of IL-8 and TNF-α during the critical phase of the disease and a significant increase in adhesion molecules were detected in both phases, but the highest levels of sVCAM-1 and sIL-2R were observed only during the acute stage of the disease. In conclusion, sIL-2R and sVCAM-1, as early markers of lymphocyte and endothelial activation, would serves as indicators of severity during the acute phase of dengue infection.

Keywords (english)

Dengue, cytokines, endothelial damage, soluble cell adhesion molecules, soluble lymphocyte markers

Resumen (español)

Varios mecanismos inmuno-patogénicos se han propuesto para explicar el incremento masivo de la permeabilidad vascular observada en las formas severas de la infección por el Virus del Dengue (DENV). El objetivo del estudio fue determinar los cambios cinéticos de mediadores inflamatorios (IL-8, TNF-α), marcadores soluble de activación linfocítica temprana (sIL-2R, sTNF-Rp75) y fracciones solubles de moléculas de adhesión celular (sICAM-1, sVCAM-1) como marcadores tempranos de severidad en pacientes con dengue. Veinte pacientes clasificados como Dengue (Dengue±Signos de Alarma,D±WS) y treinta pacientes con Dengue Severo (DS) fueron incluidos en el estudio. Suero de individuos aparentemente saludables fueron incluidos como controles. En comparación con los individuos controles, los casos con Dengue mostraron niveles de IL-8 y TNF con diferencias no significativas en la fase febril o crítica de la enfermedad; sin embargo, un incremento significativo de sICAM-1 y sVCAM-1 ocurrió en ambas fases, mientras que los niveles de sIL2R y sTNF-p75 se elevaron significativamente solo en la fase crítica de la enfermedad. En comparación con los casos con dengue y controles, los pacientes con DS

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mostraron diferencias significativas en los niveles de IL-8 y TNF-α durante la fase crítica y un incremento significativo de moléculas de adhesión en ambas fases, pero los niveles más elevados de sVCAM-1 y sIL-2R fueron observados en la fase febril. En conclusión, sIL-2R y sVCAM-1, como marcadores tempranos de activación linfocítica y endotelial, servirían como indicadores de severidad en la fase aguda de la infección por el virus del dengue.

Palabras clave (español)

Dengue, citoquinas, disfunción endotelial, moléculas de adhesión células solubles, marcadores solubles linfociticos.

Introduction

Dengue virus (DENV) infection is currently the most important arthropod-borne viral disease with more than a half of the world's population living in disease risk´s areas, with an estimated of more than 50 million cases each year(1-5). Infection caused by any of the four Dengue flaviviruses (DENV1-DENV4) (6). Dengue is a complex disease with a wide spectrum of clinical presentations, however, the majority of the cases develop mild self-limited disease defined as Dengue, without or with warning signs (D±WS, Groups A and B), whereas only a small proportion develop a serious clinical manifestations with unpredictable clinical evolution known as Severe Dengue (SD, Group C), including Shock for Dengue (7, 8). The severity of Dengue varies dependent upon host factors (age, genetic makeup of the population, ethnicity, immune status) (9-12), circulating DENV (genotype, viral load, intrinsic pathogenicity, sequence of infection, along with heterotypic cross-protection following infection) (13-16) and environment factors (hyperendemicity) (5, 17, 18).

Various hypotheses have been proposed to explain the immune pathogenesis of Severe Dengue (19-22). Among them, antibody-dependent enhancement (ADE) of infection (23) and presence of antibodies against NS1 (24), a viral protein that cross-reacting with platelets and vascular endothelium (25, 26), have an essential role. The humoral response is responsible for controlling the infection and dissemination of the dengue virus in the body. The cross-reactive nature of the antibodies leads to temporary heterotypic immunity mediated by viral E protein specific neutralizing antibodies which inhibits viral attachment and entry into host cells. Antibodies may also bind to complement proteins and promote activation of the complement cascade (27).

Massive immune activation of infected cells, leads to shift from Th1-type cytokine response in Dengue to a Th2-cytokines response in SD (28-30), that cause endothelial dysfunction, increasing vascular permeability and finally adverse effects on vascular endothelium cells (24, 31-33).

Among cytokines, tumor necrosis factor-α (TNF-α), interleukin-6 (IL6), IL-8, IL-10 and transforming growth factor-β1 and macrophage migration inhibitory factor (MIF) are associated with disease severity, and autoimmunity (29, 34-36). Some of these cytokines leads to the cellular expression and release of receptors associated with vascular activation and damage including soluble TNF-α and IL2 receptors (37-39), soluble CD8 (20,40), and up-regulation of intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1 respectively) (41-44).This effects are more important in patients with severe dengue, although it can be found at lower degrees in patients with mild disease. It has been proposed that, secondary Dengue infection induced abnormal and/or accelerated T cell responses that attack infected macrophages leading to a subsequent increased cytokine production (19, 45, 46). However, a rise of cytokines, chemokines or soluble receptors depends of which immune cells are activated, viral load, disease stage, and severity (35, 41, 47). Only few studies have correlated cytokine levels with the stage of the disease(48-50), and since severe manifestations of Dengue disease mostly develop around the time of critical stage, changes in biological markers should be analyzed in the context of the stages of the disease (51, 52). We hypothesized that dynamic changes of proinflammatory mediators (IL-8, TNF-α), soluble lymphocyte activation markers (sIL-2R, sTNF-Rp75) and soluble cell adhesion molecules (sICAM-1 and sVCAM-1) occurred during the stages and severity of the diseases. Because clinical evidence suggests that vascular activation and T cell activation may be involved in the early stage of SD, our goal was to determine the in vivo levels of pro-inflammatory cytokines, soluble adhesion molecules and lymphocyte activation markers during the acute and critical stages of DENV infection and its association with severe course of the disease.

Materials and methods

***Clinical samples*.** Paired blood samples were collected from peripheral veins of fifty (n = 50) febrile

patients during the acute (1-3 days after onset of fever, DOF, during viraemia) and critical stage (4-7days after onset of fever, DOF) of the disease. Critical phase of the disease is defined by WHO as the phase that stars around the time of defervescence, when the temperature drops to 37.5–38ºC or less (remaining below this level), usually on days 3–7 of illness. An increase of haematocrit levels can occur due to changes in endothelial permeability (7). This phase is mark by a clinically significant plasma leakage usually lasts 24–48 hours (7). Progressive leukopenia followed by a rapid decrease in platelet count usually precedes plasma leakage. At this point patients without an increase in capillary permeability will improve, while those with increased capillary permeability may become worse as a result of lost plasma volume (7). Therefore, WHO criteria of critical phase were used to select the time course division during the immune markers analysis.

DENV infection was confirmed by either in-house real time RT-PCR (53) during the acute stage and by serological tests (anti-Dengue IgM antibody by ELISA-based test) during the critical stage (54, 55) in every sample of each patient included in the study. Seven (n = 7) apparently healthy individuals, without febrile or other illnesses were included as internal controls. Sera were obtained by centrifugation at 4ºC and stored at -20ºC until the day of assay. A detailed history and physical examination was performed along with laboratory determination every 24 hours until complete recovery of patients. The study protocol was approved by ethics committee of Universidad de Los Andes and written informed consent was obtained from all patients or their guardian's prior inclusion to the study following the basic principles of the Declaration of Helsinki (56, 57).

***Determination of IL-8, TNF-α, sICAM-1, sVCAM-1, sIL-2R and sTNF-Rp75*.** Levels of IL-8 (ng/mL, Chemicon International Inc., CA, USA), TNF-α (pg./mL, Biosource International Inc., CA, USA), sICAM-1, sVCAM-1 (ng/mL, Chemicon International Inc., CA, USA), sIL-2R and sTNF-Rp75 (ng/mL, Biosource International Inc., CA, USA) were determined using commercial ELISA kits in compliance with manufacturer's directions. For each assay, samples were tested by triplicates.

***Statistical analysis*.** Data base and all statistical analyses were performed using EPI Info software (version 3.5; Center for Disease Control and Prevention, GA, USA). Results are reported as frequency of distribution, mean or standard error of the mean (± SEM). The comparison between groups was done using Student's t-test and one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. Values were considered statistically significant at a p value less than 0.05**.**

Results

Clinical and laboratory findings in patients with Dengue. Fifty patients with confirmed DENV infection were included in this study. Among them 54% (27/50) were female and 46% (23/50) male with a median age of 26 years old (SEM ± 18.20) (table 1). From the clinical standpoint, 40% (20/50) of the cases were classified as Dengue±Warning Signs (D±WS) and 60% (30/50) were recognized as SD (Table 1). Within D±WS cases (n=20), patients age range from 3–52 years-old (Mean value of 26,4 years); eight (8/20) were pediatric patients with age range from 3-11 years-old (mean value 7,1 years, SD:3,6); and twelve (12/20) adults with age range from 18-53 years-old (mean value 39,2 years, SD: 12,3). Within DS patients (n=30), age range varied from 5 months-old to 68 years-old (Mean value of 23,2 years); fifteen (15/30) were pediatric patients with age range from 0.5-16 years-old (mean value 7 years, SD:4,7); and fifteen (15/30) range from 19-68 years-old (mean value 33,5 years, SD: 15,3) (Table 1).

Clinical and laboratory characteristics of the 50 patients are shown in Table 1. At the moment of the physical examination, 85% (17/20) of patients with D±WS and 90% (27/30) of cases with SD had fever at the moment of recruitment. Rash and bleeding were a significantly finding in patients with SD (66%, 83% respectively). Rise of hematocrit (>20% from base level) were present in 96% (29/30) of patients suffering SD ($p < 0.001$). Platelets count were lower than 150.000 platelets/mm^3 in all patients included in the study, the lower counts were observed in SD patients with a mean value of 66.000/mm^3 (Table 1).

During the acute phase of the disease, viraemia was detected in 45% (9/20) of patients with D±WS and 36% (11/30) of patients with SD. During the critical stage, IgM anti-Dengue was detected in 65% (13/20) patients with D±WS and 100% (30/30) in SD cases. DENV2 was predominant in both group of patients, D±WS and SD (66.6% and 45.5% respectively). All four serotypes of DV were amplified in patients with SD, while DENV-1 was not amplified patients with D±WS (Table 1).

Elevated serum levels of IL-8, TNF-α in the critical stages of Severe Dengue cases Pro-inflammatory cytokines were determined in seventeen

Table 1. Clinical and laboratory findings in patients with dengue at the time of admission to the study

Clinical features	D±WS *n* = 20 (%)	SD *n* = 30 (%)	*p* - value
Age (years)	26,4 (3y-52y)	23,2 (5m-68y)	
Pediatrics	n=8	n=15	
(months-17 years-old)	(x=7,1, sd=3,6)	(x=7,0, SD=4,7)	0.68
Adults	n=12	n=15	
(18 years-old and more)	(x=39,2, sd=12,3)	(x=33,5, sd= 15,3)	
Fever	17 (85)	27 (90)	0.83
Rash	7 (35)	20 (66)	**0.04***
Bleeding	3 (15)	25(83)	**<0.001***
Clinical Laboratory			
Determinations	**D±WS *n* = 20 (%)**	**SD *n* = 30 (%)**	***p* - value**
Hemoglobin (>11g/dL)	4(20)	13(43)	0.12
Increased Hematocrit Level ¥	3(15)	29(96)	**<0.001***
White Blood Count(>4000xmm^3)	12(60)	17(56)	0.78
Neutrophils (>40%)	8(40)	15(50)	0.43
Lymphocytes (>20%)	5(25)	7(23)	0.83
Platelets (Mean)	149.000/mm^3	66.000/mm^3	**<0.001***
Confirmation Tests (*)			
qPCR-Universal	9(45.0)	11 (36.6)	
DENV1	0 (0.0)	2(18.1)	NA
DENV2	6 (66.6)	5 (45.4)	NA
DENV3	3 (33.3)	3 (27.2)	NA
DENV4	0 (0.0)	1 (9)	NA
IgM anti-dengue	13 (65)	30 (100)	NA

D±WS: dengue ± warning signs; SD: severe dengue; NA: non-applicable. m=month; y=years-old. ¥A rise of Hematocrit by more than 20% from the base level was considered as hemoconcentration. *p<0.05. Two-patients were positive for both qPCR and IgM-anti-dengue simultaneously. Confirmation test were performed to all patients as described in methods sectionConfirmation test were performed to all patients as described in methods section.

(N = 17) patients, 41.2% (7/17) with D±WS and 58.8% (10/17) with SD. Levels were analyzed and distributed according course and severity of the disease.

Levels of IL-8 were similar in patients with D±WS during both acute and critical phases of the disease (Mean value 0.19 and 0.30 ng/mL, respectively). In patients with SD, a significant increase of IL-8 was observed in the critical phase (Mean value 0.53 ng/mL) of the disease compared with acute phase (0.25ng/mL, p = 0.013) (figure 1a).

Regarding TNF-α determination, during the acute phase of the disease patients with SD showed lower levels of this cytokine compared with patients with D±WS and control group (Mean values 8.9 and 28.8 pg./mL respectively, p = 0.159). During the critical phase of the disease, levels of TNF-α were higher in patients with SD than in patients with D±WS (Mean values 80.2 and 8.9 pg/mL respectively p = 0.042). There were no differences in TNF-α level between patients with D±WS and control group during critical stage (figure 1b).

Differential release of endothelial cells adhesion molecules, ICAM-1 and VCAM-1, in patients with Dengue during the acute and critical stages of the disease. Soluble adhesion molecules were determined in seventeen (N = 17) patients, 41.2% (7/17) with D±WS and 58.8% (10/17) with SD. Levels were analyzed and distributed according course and severity of the disease.

Similarly, the degree of endothelial cell (EC) activation was analyzed according to the stages of the disease. Levels of sICAM-1 were significantly higher in patients with D±WS and SD compared with control group during the acute (Mean values of 48.1, 46.5, 17.1 ng/mL respectively, p = 0.001) and critical (Mean values of 50.1, 92.0, 12.4 ng/mL respectively, p <0.001) phases of the disease. However, during the critical phase of the disease, patients with SD shown higher levels of sICAM-1 compared with D±WS patients (Mean values of 50.1, 92.0ng/mL respectively, p = 0.002) (Figure 2a).

Levels of sVCAM-1 were significantly higher (p = 0.012) in patients with SD during the acute phase of the disease (Mean values of 96.5ng/mL) compared with patients classified as D±WS (Mean values of 43.4ng/mL); while levels during the critical phase of the disease were similar between both groups of patients (p = 0.268) (Figure 2b).

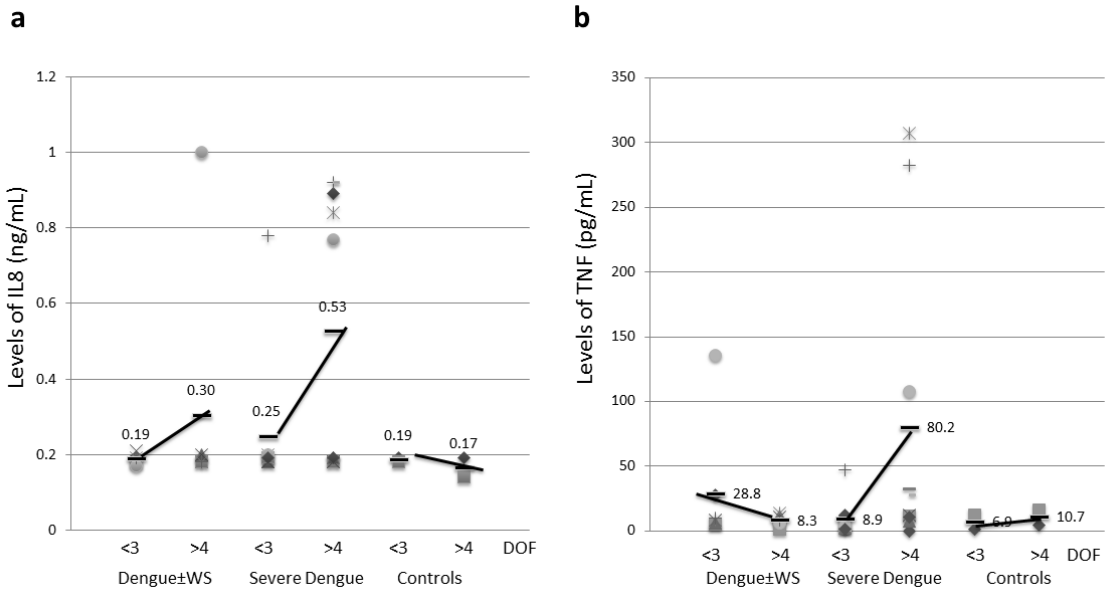

Figure 1. Levels of IL-8 **(a)** and TNF-α **(b)** in serum samples of patients with Dengue without or with warning signs (DENGUE), severe Dengue (SD) and individual controls collected during the course of the disease adjusted by the days after the onset of fever (DOF). Levels were measured in ng/ml for IL-8 and in pg./mL for TNF. Samples were performed by triplicates and black squares indicate mean value for each group

Differential increased of serum levels of sIL-2R and sTNF-Rp75 during acute and critical stages of Dengue infection. Serum samples of thirty-three (N = 33) infected patients in acute and critical phases of the disease, were used to determined levels of sIL-2R and sTNF-Rp75. From those patients, 39.4% (13/33) were clinically classified as D±WS and 60.6% (20/33) with SD.

In the acute phase of the disease, levels of sIL-2R were significantly higher in serum samples of patients with SD compared with D±WS (Mean values of 501.0 versus 298.7 ng/mL respectively, p = 0.016). Nevertheless, at the critical phase, levels of sIL-2R were higher in patients with D±WS compare with patients clinically recognized as SD (Mean values of 483,0 versus 312,4 ng/mL respectively, p = 0.022) in whom levels of this activation marker showed a significant decreased (Figure 3a).

For the soluble form of TNF receptor (sTNF-Rp75), levels were higher during the critical phase of the disease in patients with D±WS and SD compared with the acute phase. Levels of sTNF-Rp75 during the acute phase of illness were similar between patients with D±WS and SD (Mean values of 25.4 and 29.4 ng/mL respectively); while during the critical phase patients with D±WS had a significant increase compared with SD group (Mean values of 91.4 versus 48.4 ng/mL respectively, $p < 0.001$) (Figure 3b).

Discussion

We attempted to determine the dynamic changes of proinflammatory cytokines (TNF-α, IL-8), soluble endothelial cell surface proteins (ICAM-1, VCAM-1) and early activation proteins released during lymphocyte activation (sTNFR and sIL-2Rα) in serum samples from Venezuelan patients infected with Dengue Virus (DV), during the different stages of the disease, in order to correlate whether those proteins servers as early markers, predictive of severe form of the disease (SD), compared with patients with non-severe forms of Dengue. Bleeding, skin rash, hemoconcentration and thrombocytopenia were the most significant signs found in cases with severe Dengue; and all DENV serotype were circulating at the time of the study, been DENV-2 the most prevalent serotype.

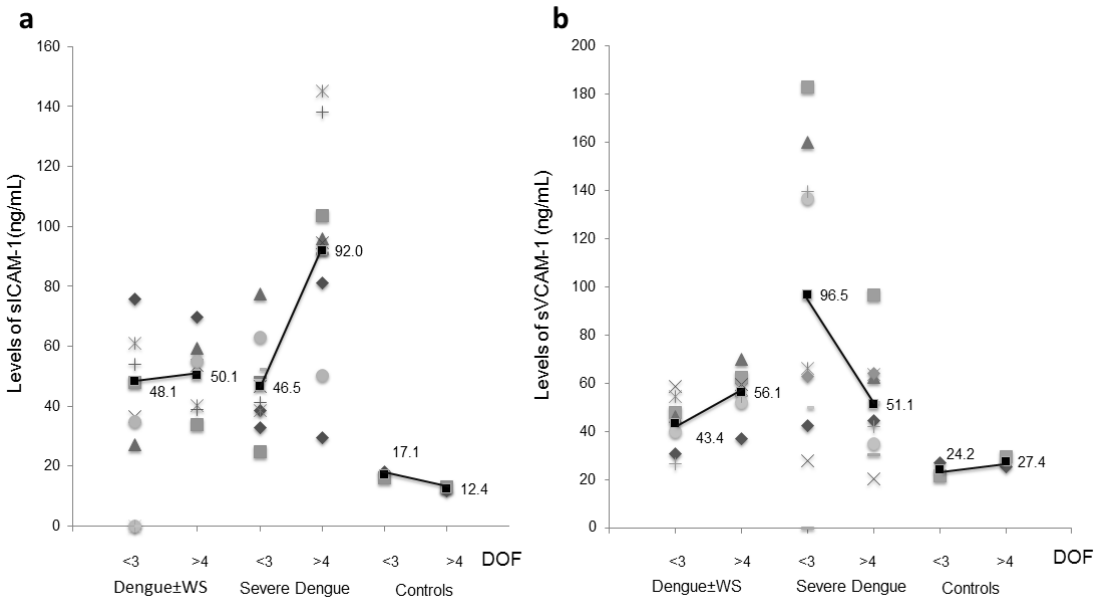

Figure 2. Levels of sICAM-1 **(a)** and sVCAM-1 **(b)** in serum samples of patients with Dengue without or with warning signs (DENGUE), severe Dengue (SD) and individual controls collected during the course of the disease adjusted by the days after the onset of fever (DOF). Levels were measured in ng/ml for both adhesion molecules. Samples were performed by triplicates and black squares indicate mean value for each group

Several mechanisms are involved in the pathogenesis of DENV research and one of the most challenger had been the identification of soluble factors that can mediate the functional changes induced in endothelial cells that are associated with the increased plasma leakage and severe forms of the disease (58). Conflicting results had been observed from the analyses of several immune markers studies in dengue infection, mainly due to differences in study design, experimental procedures, and the time of sampling during infection, which together leads to discrepant results (59).

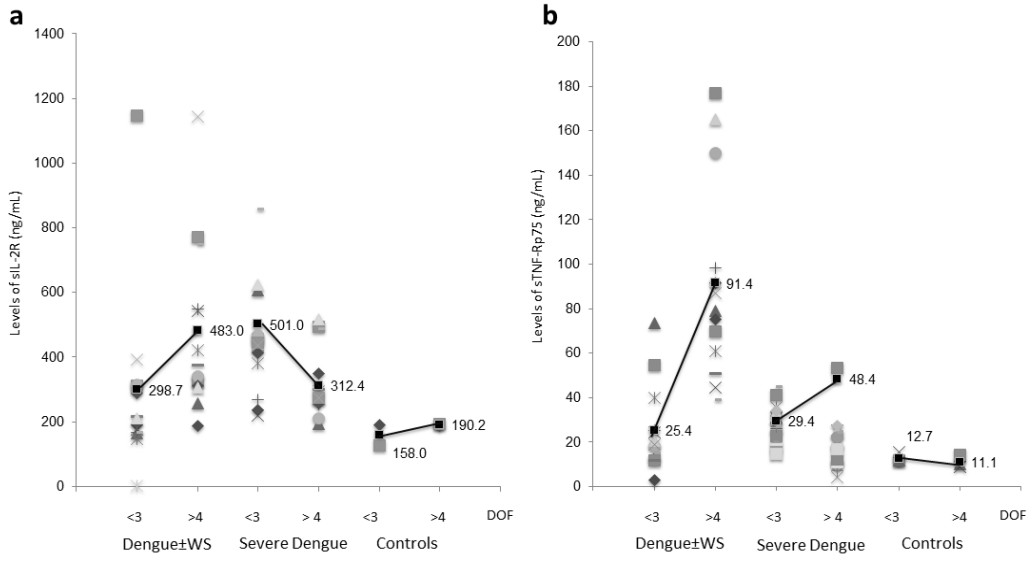

Figure 3. Levels of soluble forms of sIL-2R **(a)** and sTNF-Rp75 **(b)** in serum samples of patients with Dengue without or with warning signs (DENGUE), severe Dengue (SD) and individual controls collected during the course of the disease adjusted by the days after the onset of fever (DOF). Levels were measured in ng/ml for both soluble forms of the receptors. Samples were performed by triplicates and black squares indicate mean value for each group.

TNF-α is a potent proinflammatory cytokine with pleiotropic properties, such as antiviral effects on several viruses(60). Increased levels of TNF-α had been determined in patients with SD compared with Dengue±Warning Signs, associated with thrombocytopenia, endothelial cells activation and hemorrhagic symptoms (61, 62); along with the fact that high levels of TNF-α do not affect DENV replication(60) and inhibit intracellular mechanisms of cell survival inducing apoptosis (63). Because TNF-α has a short half-life in circulation, its levels in particular time of DENV infection do not reflect its effects during the course of infection, therefore, we analyzed its levels according to the stage of the disease. Levels of TNF-α rise in non-severe forms of Dengue during the acute stage (viraemia), and decreased during critical stage. However, a significant rise was observed at the critical stages in patients with severe forms of the disease (SD). Most cellular and animals models of immunopathology has shown that TNF-α is released from several cells from immune system (monocytes/macrophages, B and T Lymphocytes), and those cells are responsible of high circulating (endocrine) levels that may in turn, induce viral pathogenesis and, in addition, act in an autocrine or paracrine manner to modulate viral replication in DV-infected cells(64). In turn, tissue and cell damage are mediated via direct lyses of infected cells by TNF-α (63); and can be blocked by the addition of anti-TNF-α antibodies (60). Therefore, in patients with Severe Dengue accumulation of TNF, during the critical stage of DENV infection, is responsible for the induction of EC activation, capillary permeability, vascular leakage, activation of fibrinolysis system and cell apoptosis, including endothelial and T cells (59).

Despite of elevated plasma levels of TNF-α and soluble TNF receptor (sTNFR) have been reported in patients with DENV infections, the dynamics over the stages of the disease still not clear(48, 65). Considering the pathophysiological importance of sTNFR p75 for the regulation of the bioavailability of TNF-α in the body, we determined the serum levels of sTNFR p75 and TNF-α in patient with laboratory-confirmed Dengue infection by using immunoassays. We found a significant elevation of the levels of sTNF-Rp75 during the critical stage of patients with DENGUE compared with patients with SD at the same stage of the disease. Although elevated levels were observed in SD, there were not enough to mediate the biological functions of TNF-α during critical stage. Soluble forms of the two molecular species of the cell surface TNF receptors (sTNFR p55 and sTNFR p75) can reduce the activity of TNF-α but they may also enhance its function by stabilizing the active TNF-α oligomer (37). Wang L. et. al. in 2007, found that lower levels of membrane-associated receptor form (mTNFR1) expression, but not levels of TNF-α, sTNFR, or mTNFR2, correlated significantly with patients with severe forms of the disease(38). Therefore, a rapid decrease in mTNFR on the cell surface and shedding of such receptors from the cell surface may serve to transiently desensitize cells, thereby providing a mechanism for inhibition of TNF-α activity (38). Fernandez-Mestre, MT. et. al., 2004 found a significant increase of the TNF-308A allele among patients with SD compared to patients with Dengue±Warning Signs, hence patients with this allele are genetically predisposed to express higher levels of TNF-alpha(66). Shedding of this receptor would point its contribution to block the elevated levels of TNF-α secreted upon activation of monocyte/macrophage system during the early stage of the disease(37); and therefore, protect endothelial cells from damage during critical stages of the disease and may be altered in SD cases. Further studies of polymorphism of TNF-α and other proinflammatory cytokines, in Venezuelan population might elucidate the role of polymorphism in TNF levels and activities.

IL-8 is a chemokine that is abundantly produced by monocytes, EC, and hepatocytes. Liver inflammation and damage cause by Dengue in endothelial cells may lead to an increase of systemic levels, which in turn activate the coagulation system resulting in a release of IL-6 and IL-8 by monocytes. In contrast, APC-PS anticoagulation pathway downregulates production of IL-8 by endothelium cells (reviewed by (59). IL-8 has an effect on the expression of adhesion molecules and tight junction proteins (67), and may contribute to a procoagulant state during DENV infection (68). Here, we also observed increased levels of IL-8 only during the critical stage of the disease, correlated with disease severity. Upregulation of IL-8 in EC, along with other pro-inflammatory cytokines, are correlated with several phenomenon during DENV infection, including DENV replication of NS4b and NS5 genome fragments (69, 70), and stimulation by antibodies anti-NS1 (43). In contrast, Priyadarshini et.al, 2010, found an early to late post-onset day (2–5 days) of illness time trend for IL-8 levels predominantly in DHF compared with DF cases (49). Therefore, variation in the time of collection of samples by stages (acute versus critical stage) and the genetic population (Western India) may explain the early release of IL-8 during the clinical course. Further studies are needed to elucidate genetic makeup related to cytokine profile and populations.

Furthermore, serum levels of IL-8 are associated to disease severity and have the major impact during the critical stage of the disease where damage to endothelial vascular cells occurs.

Cytokines such as TNF-α and IL-8 has an effect on the expression of adhesion molecules such as ICAM-1 and VCAM-1 on endothelial cells. In this study, serum circulation of sICAM-1 were appreciably higher in patients with SD, while in Dengue±Warning Signs cases levels rise during the critical stage of the disease. Interestingly, high mean levels of sVCAM-1 were observed in the first 3 days of illness (acute stage) in SD patients. Cardier et al., 2005 found that sera from patients with acute Dengue induced an increase in ICAM-1 expression on HMEC-1. This effect was greater with samples from the acute febrile phase than with samples from the convalescent phase of the disease (61); and also found a significant increase in plasma levels of sICAM-1 and sVCAM-1 in patients with severe Dengue (33). Koraka et al., 2004 found elevated levels of VCAM-1 in children with acute DENV infection associated with disease severity, and the time post infection (acute vs. convalescent phase) and not with age, sex, or previous exposure of the patients to Dengue infection (47). Therefore, sVCAM-1 would be an important early marker to predict the occurrence of severe cases of Dengue.

IL-2 is considered a marker of immune system activation and proliferation, since appear before other cell surface determinants and induce a predominant T cells proliferation, B cell activation and synthesis of proinflammatory cytokines such as IFN-γ and TNF-α, which in turn can induce EC activation and damage (39). Following mononuclear cell activation a soluble form of the alpha-chain of IL-2R (sIL-2Rα or CD25) may be released by proteolytic cleavage at the cell surface (39). The sIL-2R has been shown to be present in higher amounts, in sera from subjects affected by several pathological condition such as neoplasia disease, autoimmunity and several infection including virus (HIV, measles and hepatitis) and parasites (39). Valero N, et. al., 2008 found an increased levels of sIL-2R and sICAM- in patients with DF and DHF, particularly, sIL-2R were related to the different grades of Dengue severity(44). In contrast, we observed increases levels of sIL-2R in patients with Dengue compared to control; however, it was a significant release of sIL-2R in the early stages of the disease in patients with Severe Dengue compare with patients with Dengue±Warning Signs, in which higher levels were observed only during the critical stage of the disease. Kurane I, et.al., in 1991 found in Thailand children (4-14 years-old) that levels of sIL-2R, sCD4, and sCD8 were higher in severe forms of Dengue (DHF) than in DF on days 3-4 after the onset of fever, initiating critical stage of the disease (40). These authors did not find differences between the levels of lymphokines between patients with dengue fever and dengue hemorrhagic fever, however, most of the patients admitted in the study were enrolled when symptoms were already severe or rapidly worsening and some of the immune response were decreased at the moment of enrollment (40). In our cohort increasing levels of IL2Rα were significant higher during days 1-3 after de onset of fever, previous to the critical stage and to the development of plasma leakage and severe dengue. Other viruses' infection such as HIV, Hepatits B, infectious mononucleosis and measles have been shown an increased levels of sIL2R in the early stages of the infection, even before the onset of symptoms, however, only in HIV infection a correlation between infection and severity have been shown, since levels of sIL2R in seropositive subject are predictive for development of AIDS (39). Therefore, high levels of soluble receptor for IL-2α (CD25) reflect early immune system activation through lymphocytes cells that leads to synthesis of proinflammatory cytokines and EC activation, and could be consider an early predictive marker and an indicator of onset of severe forms of the disease.

Further evaluation in a larger population may be required, since in our study it was a limited sampling size capability for the multiple analyses that were performed to each patient (qPCR, serology, cytokine determination, hemograms and others).

In conclusion, here we highlight insights concerning to soluble factors (cytokines and receptors) associated to endothelial cell dysfunction, hallmarks of plasma leakage associated to severe forms of Dengue. We found an early increased of sIL2-R and sVCAM-1 expression in serum samples significantly associated to severe forms of Dengue during the early stages of the disease, which could operate as markers of severity in patients progressing to complicated forms of Dengue. Cytokines such as TNF-α and IL-8 has an important effect on the expression of adhesion molecules such as ICAM-1 and VCAM-1 on endothelial cells. A significant elevation of the levels of sTNF-Rp75 during the critical stage of patients with non-complicated forms of Dengue infection but no in severe forms rise its contribution to block the elevated levels of TNF-α secreted upon activation of monocyte/macrophage system during the early stage of the disease, protecting endothelial cells from damage during critical stages of the disease. Finally, cytokine profile identified in patients with Dengue may represent a

valuable tool for the characterization of patient groups at risk for developing severe disease.

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Conflict of interest statement

The authors declare that no conflicts of interest exist.

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