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Original Article

Role of cytokines, chemokines, C3a, and mannose-binding lectin in the evolution of the chikungunya infection

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Abstract: The pathogenesis of the severity of chikungunya infection is not yet fully understood. Objective: To assess the role of the cytokines/chemokines and system of complement in the evolution of chikungunya infection. Methods: In both acute and chronic phases, we measured the serum levels of 12 cytokines/chemokines and two complement mediators: mannose-binding lectin (MBL) and C3a, in 83 patients with chikungunya infection and ten healthy controls. Results: During the acute phase, 75.9% of the patients developed musculoskeletal disorders, and in 37.7% of them, these disorders persisted until the chronic phase. In general, patients had higher levels of cytokines than healthy controls, with significant differences for IFN-γ, IL-6, IL-8, IL-10, and MIP-1. Most cytokines exhibited a downward trend during the chronic phase. However, only IL-10, and MIP-1 levels were significantly lower in the chronic phase. Additionally, these levels never decreased to concentrations found in healthy controls. Moreover, MBL levels were significantly higher in the acute phase compared with the chronic phase. C3a levels were significantly higher in patients with musculoskeletal disorder compared with patients without it, in both acute-phase 118.2 (66.5-252.9), and chronic phase 68.5 (64.4-71.3), $P < 0.001$. Interestingly, C3a levels were significantly higher when patients had a severe disease version. Besides, in the acute phase, C3a levels were higher in patients that suffer arthritis as opposed to when they suffer arthralgia, 194.3 (69.5-282.2), and 70.9 (62.4-198.8), $P = 0.013$, respectively. Conclusions: Our results showed an immunological response that persisted until the chronic phase and the role of the complement system in the severity of the disease.

Keywords: Chikungunya virus, musculoskeletal disorder, disease severity, cytokines, C3a, mannose-binding lectin

Introduction

Chikungunya virus (CHIKV) is an arthritogenic virus that belongs to the *Togaviridae* family, genus Alphavirus, transmitted by *Aedes* mosquito bites. CHIKV emerged in the Americas in Saint Martin in 2013, and has currently spread to over 100 countries [1].

Most of the infected patients present with fever, headache, nausea, vomiting, myalgias, exanthema, and severe polyarthralgia/polyarthritis that are debilitants. This disease is generally self-limiting. Nevertheless, musculoskeletal manifestations can persist for months and even years, impacting the patients' quality of life and the country's economy [2, 3].

The prevalence of patients who develop persistent musculoskeletal manifestations after three months of suffering from acute infection can range between 4.1% to 80% [4-8]. In addition to arthralgias, the musculoskeletal manifestations reported are inflammatory arthritis, synovitis, tenosynovitis/enthesitis and bursitis [9]. However, the pathophysiology of joint pain is still uncertain. One hypothesis is that the severe immune response in the post-viremia phase may be related to the severity of the infection and the development of chronic musculoskeletal manifestations [10-13]. Thus, the excessive production of cytokines driven by the activation of different cell populations in the early stages of the infection contributes to the progression of the disease.

Interleukin (IL)-1b, IL-6, IL-8, IL-12, interferon (IFN)-γ and MCP-1 (monocyte chemoattractant protein) [13], as well as IL-7 and IL-15, are involved in the acute phase of inflammation. On the other hand, cytokines IL-6 and GM-CSF (granulocyte-monocyte colony-stimulating factor) and chemokines IL-8, MCP-1 and MIP-1 (macrophage inflammatory protein-1) are associated with the persistence of arthralgia in CHIKV [11, 12], though with inconclusive results. Furthermore, high levels of IL-10 have been detected in CHIKV recovered patients [14].

In addition, the role of the mannose-binding lectin (MBL) in the severe clinical evolution of the Ross River virus infection (RRV), another arthritogenic alphavirus, has been demonstrated [15]. MBL, through its binding to the microbial surface, is involved in the activation of the lectin pathway, one of three pathways by which the complement system can be activated. The activation of this cascade leads to the production of various fragments derived from component 3 (C3). One of these fragments is C3a, generated from the cleavage of C3 by C3-convertase. C3a is considered an anaphylatoxin responsible for mediating the local inflammatory process [16]. However, the role of the mannose-binding lectin and C3a have been rarely studied in CHIKV disease. To understand how the immune system is involved in this infection's pathogenesis, especially in triggering the musculoskeletal disorder in the chronic phase, serum levels of some cytokines and immunological mediators of the complement system were measured in patients with CHIKV infection.

Materials and methods

Type and area of study

A longitudinal study was carried out between January 2016 and September 2018 in the following municipalities of Colombia: Medellín, the capital of the department of Antioquia, with 2,400,000 inhabitants; Apartadó, a municipality of the department of Antioquia, with 178,257 inhabitants; Ibagué, the capital of the department of Tolima, with 159,268 inhabitants; and Villavicencio, the capital of the department of Meta, with 551,212 inhabitants.

Study populations

The study populations were two: 1) Patients with CHIKV infection (n = 83) recruited in health institutions of the municipalities described above, and 2) Healthy controls (n = 10).

Inclusion criteria

(1) Patients with CHIKV infection

• Patients with at least 15 days of evolution of fever and arthralgia unexplained by other medical conditions.

• Patients with a laboratory diagnosis of CHIKV infection.

Exclusion criteria

• Participants with malaria confirmed by thick smear were excluded.

(2) Healthy controls

• Healthy participants in whom IgM antibodies against CHIKV were not observed.

Samples

Two serum samples were taken from each patient at different periods of infection. The first sample was taken during the acute phase of the disease, up to 15 days after the onset of symptoms, and the second sample, during the chronic phase, three months after the start of symptoms. Samples were stored at -80°C until their processing.

Diagnosis of CHIKV infection

In the acute phase sample was confirmed by detecting specific IgM antibodies against the chikungunya virus using a Novalisa® Chikungunya IgM μ-capture - ELISA (NovaTec Immunodiagnostica GMBH, Dietzenbach, Germany) commercial kit, which was processed according to the manufacturer's instructions. Additionally, in patients between one and five days after the onset of symptoms, qRT-PCR was performed for viral genome detection, using the previously described technique [17].

Measurement of immunological mediators

The measurement of immunological mediators was carried out in both serum samples (acute

and chronic phases of the disease). C3a and MBL levels were measured using a Human Complement C3a ELISA Kit (Novus biologicals® - Biotechne®, Canada) and a Quantikine® ELISA Human MBL Immunoassay kit (R & D Systems® - biotechne®, USA), respectively. The serum levels of the following cytokines, IFN-γ, TNF-α, IL1Ra, IL-4, IL-6, IL-8, IL-10, IL-12, MCP-1, RANTES, GM-CSF, and MIP-1, were processed using commercial enzyme-linked immunosorbent assay kits (Sigma-Aldrich®, Saint Louis, MO, USA). All tests were made following the manufacturer's instructions.

Evaluation of the presence and severity of the musculoskeletal disorder

A physician evaluated the patients suffering from CHIKV in the acute and chronic phases. A patient was considered to have the musculoskeletal disorder (MSD) if they presented with localized pain and/or swelling in a joint group [18]; if this happened during the acute phase, it was named "presence". If this happened during the chronic phase, it was called "persistence". The severity of the MSD was assessed through: 1) the presence of arthritis (joint pain and swelling) or arthralgia (only joint pain); 2) pain measurement by the visual analog scale (VAS), and 3) measuring the disability index with the "Health Assessment Questionary" (HAQ). The patients were divided according to their pain into two categories: non-severe pain (scores 1 to 5) and severe pain (scores 6 to 10). They were also classified according to their functional disability in two categories: non-severe functional disability (scores 0 to 1) and severe functional disability (scores 2 to 3).

Definition of disease severity

If a patient, during the acute phase, presented at least two of the following hematological findings: thrombocytopenia < 100,000 cells/mm^3, elevated levels of alanine transaminase (ALT) or aspartate transaminase (AST) > 50 IU/L, and elevated C-reactive protein (CRP) > 3 mg/L, it was considered a severe version of the disease.

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences, SPSS (SPSS®, version 22, Inc.01., Chicago, ILL). Quantitative data were expressed as the median (interquartile range), and the qualitative data were expressed as a proportion. The Mann-Whitney test performed a statistical comparison of the level of immunological mediators between the groups. We compared the acute and chronic phases utilizing the Wilcoxon test. The cytokines levels were correlated using Pearson's correlation analysis. A *P*-value < 0.05 was considered significant.

Ethics

All subjects voluntarily signed the informed consent form, previously approved by the Ethics Committee of the Universidad CES (Medellin, Colombia), registered with number 445, which is under the ethical standards of the Helsinki Declaration of 2008 and the conditions provided by the Health Ministry of Colombia.

Results

All the 83 patients included in this study were positive for IgM antibodies to CHIKV; three were also positive for RT-PCR. The age median (IQR) of the patients was 34 (18-49) years, and 57 (68.7%) were females. The median (IQR) in days between the symptom onset and the date of evaluation during the acute phase was 6 (4-11), and the median (IQR) in months between the date of symptom onset and the date of the evaluation in the chronic phase was 3.7 (2.5-5.10). The age median (IQR) of the ten healthy controls was 32 (21.3-44.8) years, and six (60.0%) of them were men.

Clinical and laboratory findings

The most frequent clinical findings were fever, headache, myalgia/arthralgia, asthenia, anorexia, back pain, and rash. Nevertheless, other systems were affected, such as the gastrointestinal tract (diarrhea and vomiting), and the respiratory tract (cough and sore throat). Furthermore, elevated C-reactive protein (CRP) was observed in 45.3% of the patients (**Table 1**).

Frequency of musculoskeletal disorder

During the acute phase, the presence of MSD in 74.7% (62/83) of the patients was observed. MSD persisted in 33.7% (28/83) of them during the chronic phase. In this phase, the fre-

Table 1. Clinical and laboratory findings of patients with CHIKV infection (n = 83)

Signs and symptoms	n	%
General		
Fever	71	85.5
Headache	67	80.7
Myalgia/arthralgia	65	78.3
Asthenia	64	77.1
Chills	60	72.3
Back pain	40	48.2
Conjunctivitis	21	25.3
Dermatological		
Rash	32	38.6
Pruritus	26	31.3
Gastrointestinal		
Anorexia	41	49.4
Diarrhea	25	30.1
Vomiting	24	28.9
Respiratory		
Cough	31	37.3
Sore throat	28	33.7
Hemorrhagic symptom		
Petechiae	9	10.8
Epistaxis	4	4.8
Bleeding gums	4	4.8
Biological parameters		
Anemia (Hemoglobin (< 10 g/dL))*	4	6.9
Thrombocytopenia (< 100,000 cells/mmˆ3)*	8	13.8
Lymphocytopenia (< 1 × 10ˆ9/L)*	6	10.3
Elevated ALT (> 50 IU/L)**	10	13.3
Elevated AST (> 50 IU/L)**	13	17.3
Elevated CPR (> 3 mg/L)**	34	45.3

*n = 58 patients are with data. **n = 75 patients with data.

quency of persistence was significantly lower in males compared with females (15.4% vs. 42.1, P = 0.02), and it was significantly higher in patients > 30 years compared to ≤ 30 years of age (45.7% vs. 18.9%, P = 0.012) (**Table 2**).

Frequency of the severity of the musculoskeletal disorder

In the acute and chronic phases, the most common MSD found was arthralgia, 61.3% and 71.4%, respectively. Arthritis (pain and swelling) was observed in 38.7% of the patients in the acute phase. This finding persisted in 28.6% of the patients in the chronic phase. Severe pain was reported in 51.6% of the MSD patients in the acute phase, which decreased to 28.6% (8/28) in the chronic phase. We observed severe dysfunctionality in 21.0% (13/62). Only one female > 30 years old in the chronic phase still presented with this degree of dysfunctionality. None of the severe findings of the MSD (arthritis, severe pain, and severe dysfunctionality) showed significant differences by sex or age group in either the acute or chronic phase (**Table 3**).

Frequency of disease severity

In 20% (15/75) of the patients, disease severity was observed because they presented at least two of the following biochemical/laboratory features: thrombocytopenia < 100,000 cells/mm^3, elevated levels of alanine transaminase (ALT), or aspartate transaminase (AST) > 50 IU/L, and C-reactive protein (CRP) level greater than 3 mg/L.

Comparison of serum levels of immunological mediators between both CHIKV patients in the acute and chronic phase and healthy controls

Most of the serum levels in immunological mediators were higher in the acute phase than the chronic phase, with significant differences for MBL, IL-10, and MIP-1. Contrastingly, C3a, MCP-1, and RANTES remained high in both phases. Serum levels of the immunological mediators were higher in patients with CHIKV infection than in healthy controls in both acute and chronic stages, with significant differences for IFN-γ, IL-6, IL-8, IL-10, and MIP-1. C3a and RANTES levels were more elevated in healthy controls (**Table 4**). Serum levels of IL-4 and IL1Ra were not detectable in 77.1% and 72.3% of the patients, respectively, or their levels were too low (data not shown).

Serum levels of immunological mediators according to the presence/persistence of musculoskeletal disorder (MSD) in the acute phase and chronic phase

In the acute phase, C3a, IL-8, MIP-1, and MCP-1 were more elevated in patients with MSD than patients without it, with significant differences for C3a and MCP-1. In contrast, IL-6 serum levels were significantly higher in patients without musculoskeletal disorders. In the chronic

Table 2. Frequency of musculoskeletal disorder in acute phase and chronic phase of patients with CHIKV infection according sex and age

Variables	Acute phase			Chronic phase		
	Presence of MSD	OR (95% CI)	*P* value*	Persistence of MSD	OR (95% CI)	*P* value*
Sex						
Male (n = 26)	22 (84.6%)	2.33 (0.69-7.81)	0.186	4 (15.4%)	0.250 (0.08-0.82)	0.024
Female (n = 57)	40 (70.2%)			24 (42.1%)		
Age						
> 30 years (n = 46)	36 (78.3%)	1.52 (0.56-4.11)	0.406	21 (45.7%)	3.6 (1.31-9.85)	0.012
≤ 30 years (n = 37)	26 (70.3%)			7 (18.9%)		
Total (n = 83)	62 (74.7%)			28 (33.7%)		

OR: Odds Ratio; 95% CI: Confidence interval; *Chi square test.

Table 3. Type of severity of musculoskeletal disorder in acute phase and chronic phase of patients with CHIKV infection according sex and age

		Acute phase							
Type of severity	Total n = 62	Sex				Age (years)			
		Male n = 22	Female n = 40	OR (95% CI)	*P* value*	> 30 n = 36	≤ 30 n = 26	OR (95% CI)	*P* value*
Arthralgia	38 (61.3%)	15 (68.2%)	23 (57.5%)	0.63 (0.21-1.88)	0.586	20 (55.6%)	18 (69.2%)	1.80 (0.62-5.20)	0.304
Arthritis	24 (38.7%)	7 (31.8%)	17 (42.5%)	0.634 (0.21-1.88)	0.586	16 (44.4%)	8 (30.8%)	1.80 (0.62-5.20)	0.304
Severe pain	32 (51.6%)	8 (36.4%)	24 (60.0%)	0.381 (0.13-1.11)	0.111	21 (58.3%)	11 (42.3%)	1.91 (0.68-5.30)	0.303
Severe disability	13 (21.0%)	3 (13.6%)	10 (25.0%)	0.474 (0.12-1.94)	0.348	7 (19.4%)	6 (23.1%)	0.81 (0.21-2.75)	0.760
		Chronic phase							
Type of severity	Total n = 28	Male n = 4	Female n = 24	OR (95% CI)	*P* value*	> 30 n = 21	≤ 30 n = 7	OR (95% CI)	*P* value*
Arthralgia	20 (71.4%)	2 (50.0%)	18 (75.0%)	3.00 (0.34-26.2)	0.555	15 (71.4%)	5 (71.4%)	1.0 (0.15-6.64)	1.00
Arthritis	8 (28.6%)	2 (50.0%)	6 (25.0%)	3.00 (0.34-26.2)	0.555	6 (28.6%)	2 (28.6%)	1.00 (0.15-6.64)	1.00
Severe pain	8 (28.6%)	1 (25.0%)	7 (29.2%)	0.810 (0.71-9.18)	1.00	5 (23.8%)	3 (42.9%)	0.417 (0.07-2.52)	0.371
Severe disability	1 (3.6%)	0 (0.0%)	1 (4.2%)	NC**	NC**	1 (4.8%)	0 (0.0%)	NC**	NC**

OR: Odds Ratio; 95% CI: Confidence interval; *Chi square test, **NC: Not calculable.

Table 4. Comparison of MBL, C3a and cytokines levels in patient with CHIKV infection and healthy controls in acute and chronic phases n = 83

Mediator	CHIKV patients		*P* value* AP vs. CP	Healthy controls (HC) Median (IQR) pg/μL	*P* value** CHIKV patients (AP) vs. HC
	Acute phase (AP) Median (IQR) pg/μL	Chronic phase (CP) Median (IQR) pg/μL			
MBL	2.3 (1.1-3.8)	1.7 (0.9-3.1)	0.013	1.7 (1.0-2.2)	0.281
C3a	71.1 (65.9-218.9)	72.0 (65.5-198.6)	0.203	147.0 (129.1-184.4)	0.110
IFN-γ	65.0 (31.0-143.7)	54.8 (24.2-123.2)	0.091	9.7 (0.0-38.3)	0.003
TNF-α	11.4 (1.9-26.5)	13.1 (2.9-28.9)	0.930	0.0 (0.0-58.8)	0.119
IL-6	25.8 (0.34-58.3)	22.2 (4.9-52.9)	0.533	0.0 (0.0-0.4)	< 0.001
IL-12	1.6 (0.0-6.1)	2.5 (0.0-14.1)	0.096	0.0 (0.0-7.6)	0.089
IL-10	28.6 (14.7-51.4)	17.1 (10.4-26.1)	< 0.001	7.7 (5.5-10.5)	< 0.001
IL-8	74.4 (23.8-264.9)	44.5 (14.7-317.1)	0.882	7.8 (4.9-23.9)	< 0.001
MIP-1	21.6 (5.3-50.1)	9.5 (0.0-25.4)	0.004	0.0 (0.0-1.6)	< 0.001
MCP-1	197.9 (132.3-325.5)	197.7 (119.9-305)	0.172	135.1 (61.9-272.1)	0.114
RANTES	5201.4 (4480.7-10478.9)	5390.8 (4734-8451.4)	0.499	6973.1 (2426.3-13174.9)	0.926
GS-CSF	0.00 (0.00-0.901)	0.00 (0.00-1.39)	0.375	0.00 (0.00-0.48)	0.510

*Wilcoxon test; **Mann-Whitney test.

phase, serum levels of C3a and RANTES were significantly higher in patients with persistence of MSD. On the other hand, IFN-γ, IL-6, IL-8, IL-10, and MIP-1 were considerably higher in

patients without persistence of MSD than in patients with persistence of MSD (**Table 5**).

Serum levels of immunological mediators according to the severity of the MSD

C3a, IL-8, IL-10, IL-12, MIP-1, MCP-1, and RANTES levels were higher in patients with arthritis than patients with arthralgias, with significant differences in C3a and IL-12. In this same phase, C3a, IL-8, IL-10, IL-12, and IFN-γ were more elevated in patients with a severe functional disability than in patients with non-severe functional disability. Concerning the pain level, the levels of C3a, IL-8, and L-12, although higher in patients with severe pain, were found to have no significant differences (**Figure 1**). TNF-α levels were significantly lower in patients with severe pain than in non-severe pain, 15.2 (6.4-32.9) vs. 4.5 (0.0-20.7), P = 0.033. During the chronic phase, in the eight patients with arthritis, some of the cytokines in the acute phase (IL-12, MIP-1, MCP-1, and RANTES) presented higher levels, which were not significant. Moreover, in this same phase, serum levels of TNF-α were significantly lower in patients with arthritis compared with patients with arthralgias, 0.0 (0.0-2.2) vs. 15.0 (3.2-57.3), P = 0.004, and in patients with severe pain compared to those who did not present with severe pain, 12.3 (0.7-57.3) vs. 0.0 (0.0-4.7), P = 0.038.

Serum levels of immunological mediators according to the severity of the disease in acute phase

In patients with the severe version of the disease, C3a, IFN-γ, IL-8, IL-10, MIP-1, MCP-1, and RANTES were more elevated than in non-severe cases, with C3a and MCP-1 being significantly different.

Correlations

In this study, during the acute phase of the infection, a robust positive correlation between IL-6 and MIP-1 (r = 0.907; P < 0.001) and a moderate positive correlation between IL-12 and IFN-γ (r = 0.743; P < 0.001) levels were observed. During the chronic phase, a robust positive correlation was observed between IL-6 and MIP-1 (r = 0.923; P < 0.001). In patients with MSD presence in the acute phase, we observed a moderated positive correlation between IL-6 and MIP-1 (r = 0.660; P < 0.001). In patients with MSD persistence in the chronic phase, a moderated positive correlation between MCP-1 and TNF-α (r = 0.700; P < 0.001) was found (**Figure 2**).

Discussion

The clinical signs of severe joint pain from CHIKV infection may last long after the infection. However, there is still no clarity on the causes. Therefore, in this study, we assessed serum levels of some cytokines and two complement system mediators in individuals with CHIKV infection according to the evolution of the disease. The clinical manifestations found were consistent with the results of other studies [19-23].

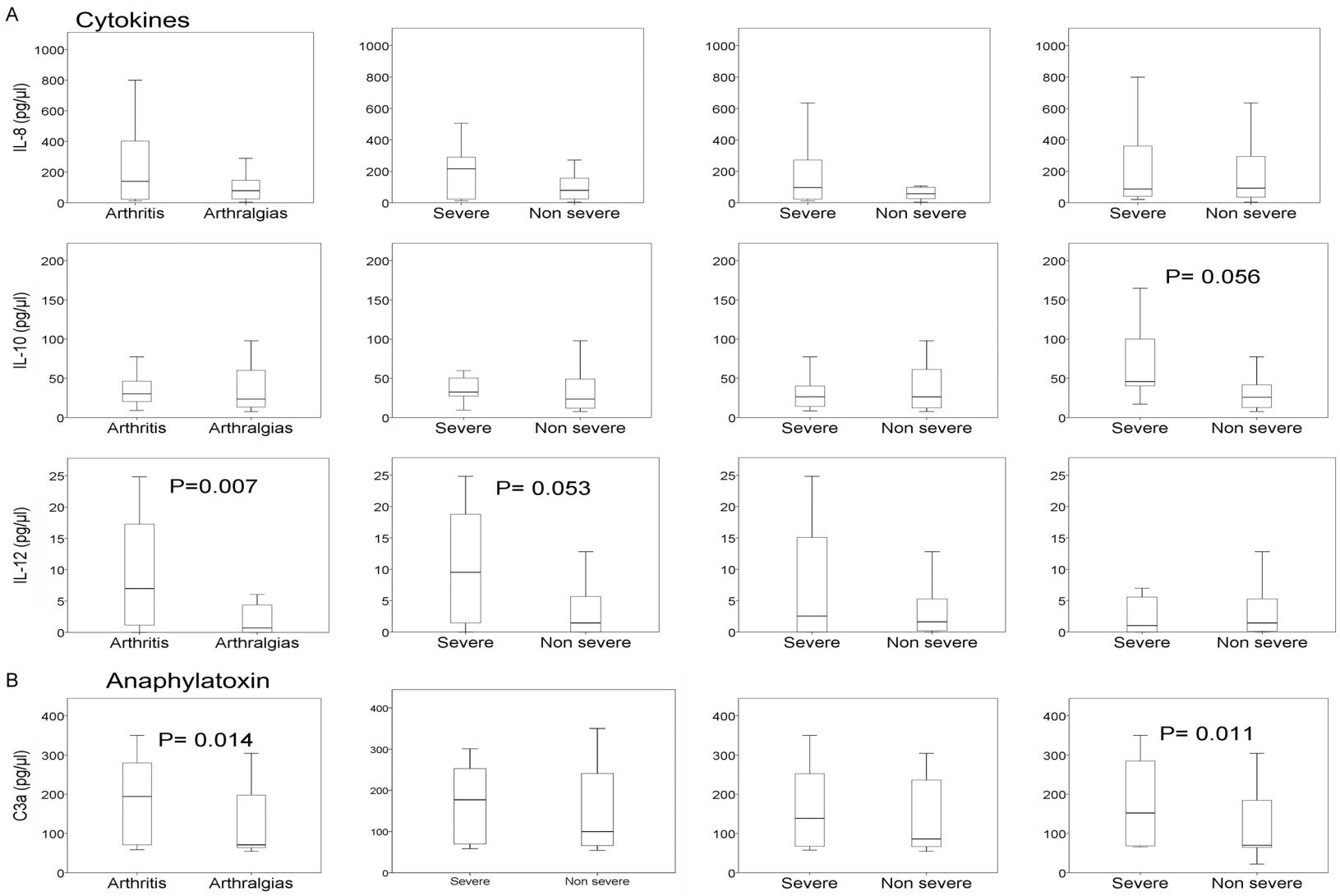
The cytokine profile in our patients during CHIKV infection in the acute phase coincided with an innate immune response, with high production of proinflammatory cytokines, in agreement with the previous reports [10, 13, 14, 24-27]. These cytokines are dependent on the early activation of Th1 and Th2, which has also been observed in other studies [13]. Consistent with Suhrbier et al., we observed that the serum levels of cytokines IFN-γ, IL-6, IL-8, IL-10, and MIP-1, were elevated in patients in the acute phase. They also remained high in the chronic phase than in healthy controls [28]. These findings could be explained by the CHIKV persistence in inflammatory cells infiltrating the joint with a high viral load in the acute phase [9, 10, 14, 19]. Interestingly, IL-10 (Th2 cytokine) levels were significantly higher in the acute phase of CHIKV patients compared with the chronic phase, and healthy controls, indicating an anti-inflammatory response, in line with previous studies [13, 14].

It is well known that the musculoskeletal disorder, a consequence of CHIKV infection, has an immunological origin [28-30], which could be related to the presence of CHIKV antigens and CHIKV RNA in joint tissue [19, 25]. In this study, presence of musculoskeletal disorder was observed in 74.7% of patients in the acute phase, and it persisted for at least three months in 37.7%, as reported by other authors [5, 7, 8, 31, 32]. When comparing the presence of the musculoskeletal disorder in patients in the acute phase, C3a, IL-8, MIP-1, and MCP-1 levels, were more elevated. In the chronic phase,

Table 5. Comparison of MBL, C3a and cytokines levels in patient with CHIKV infection according to presence/persistence of musculoskeletal disorder in acute and chronic phases

Mediator	Acute phase (AP) Musculoskeletal disorder Median (IQR) pg/μL			Chronic phase (CP) Musculoskeletal disorder Median (IQR) pg/μL			*P* value** Presence (AP) vs. Persistence (CP)
	Presence n = 62	No presence n = 21	*P* value* Presence/No presence	Persistence n = 28	No persistence n = 55	*P* value* Persistence/No persistence	
MBL	2.3 (1.2-3.8)	2.5 (1.0-3.7)	0.853	1.4 (1.0-2.9)	1.9 (0.9-3.5)	0.398	0.425
C3a	122.7 (66.6-253.3)	68.5 (64.5-71.2)	0.022	190.9 (72.5-248.2)	68.4 (63.2-164.1)	< 0.001	0.501
IFN-γ	59.4 (18.3-139.7)	72.1 (47.8-183.7)	0.166	31.0 (1.2-72.6)	61.8 (40.6-150.3)	0.005	0.101
TNF-α	11.0 (0.0-25.8)	12.0 (3.7-28.1)	0.467	5.3 (0.0-44.7)	14.6 (4.7-27.5)	0.198	0.502
IL-6	12.8 (0.0-45.0)	47.5 (23.5-94.9)	0.002	7.9 (2.4-32.8)	23.0 (8.6-77.6)	0.004	0.510
IL-12	1.8 (0.0-9.7)	1.4 (0.1-2.7)	0.496	0.3 (0.0-14.1)	4.6 (0.5-14.1)	0.090	0.398
IL-10	26.4 (13.5-51.4)	34.3 (19.6-60.4)	0.341	11.6 (7.9-19.5)	20.8 (14.4-30.0)	0.001	0.002
IL-8	82.1 (23.2-243.8)	67.9 (34.9-781.6)	0.516	20.4 (10.5-41.3)	104.7 (20.0-742.0)	0.002	0.112
MIP-1	23.9 (3.1-50.2)	17.8 (8.7-49.2)	0.963	3.4 (0.0-13.7)	16.1 (0.0-36.6)	0.008	0.002
MCP-1	229.0 (148.8-342.5)	144.7 (106.9-193.3)	0.008	223.7 (124.8-352.8)	176.1 (119.9-268.4)	0.149	0.349
RANTES	5153.0 (4495.8-10923.1)	5201.4 (4401.9-9539.6)	0.554	7721.1 (4759.8-14824.1)	5226.1 (4676.9-6965.7)	0.037	0.918

*Mann-Whitney test; **Wilcoxon test.


A
Cytokines
IL-8 (pg/µl)
IL-10 (pg/µl)
IL-12 (pg/µl)
P=0.007
P= 0.053
P= 0.056
B
Anaphylatoxin
C3a (pg/µl)
P= 0.014
P= 0.011
Arthritis
Arthralgias
Severe
Non severe

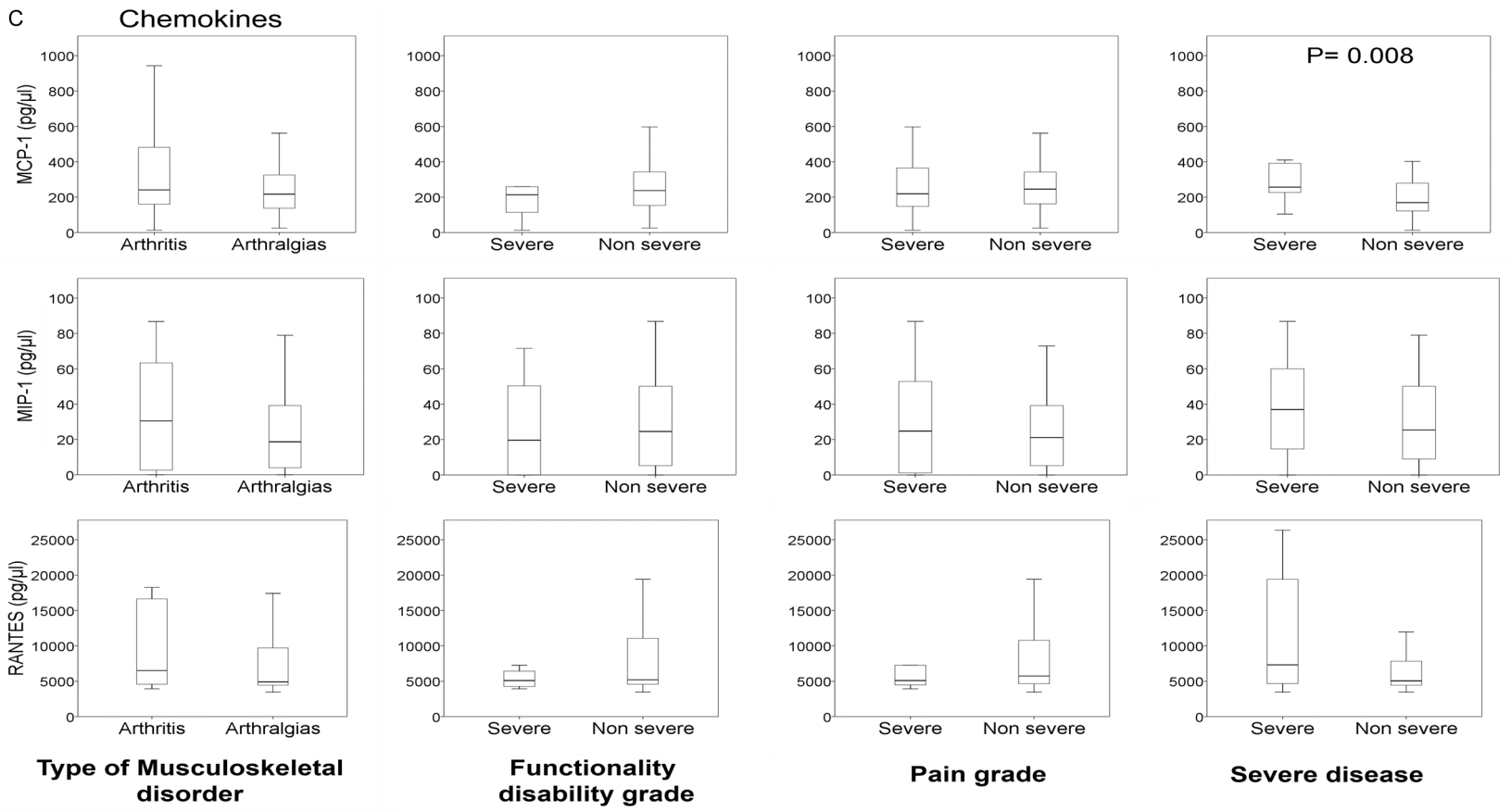


Figure 1. Serum levels of (A) Cytokines, (B) Anaphylatoxin and (C) Chemokines, in the acute phase of CHIKV infection, according to type of arthritis n = 24, arthralgia n = 38; degree of functional disability (severe n = 13, non-severe n = 49) and pain grade (severe = 32, non-severe = 30) and severity of the disease (severe n = 15, non-severe n = 60).

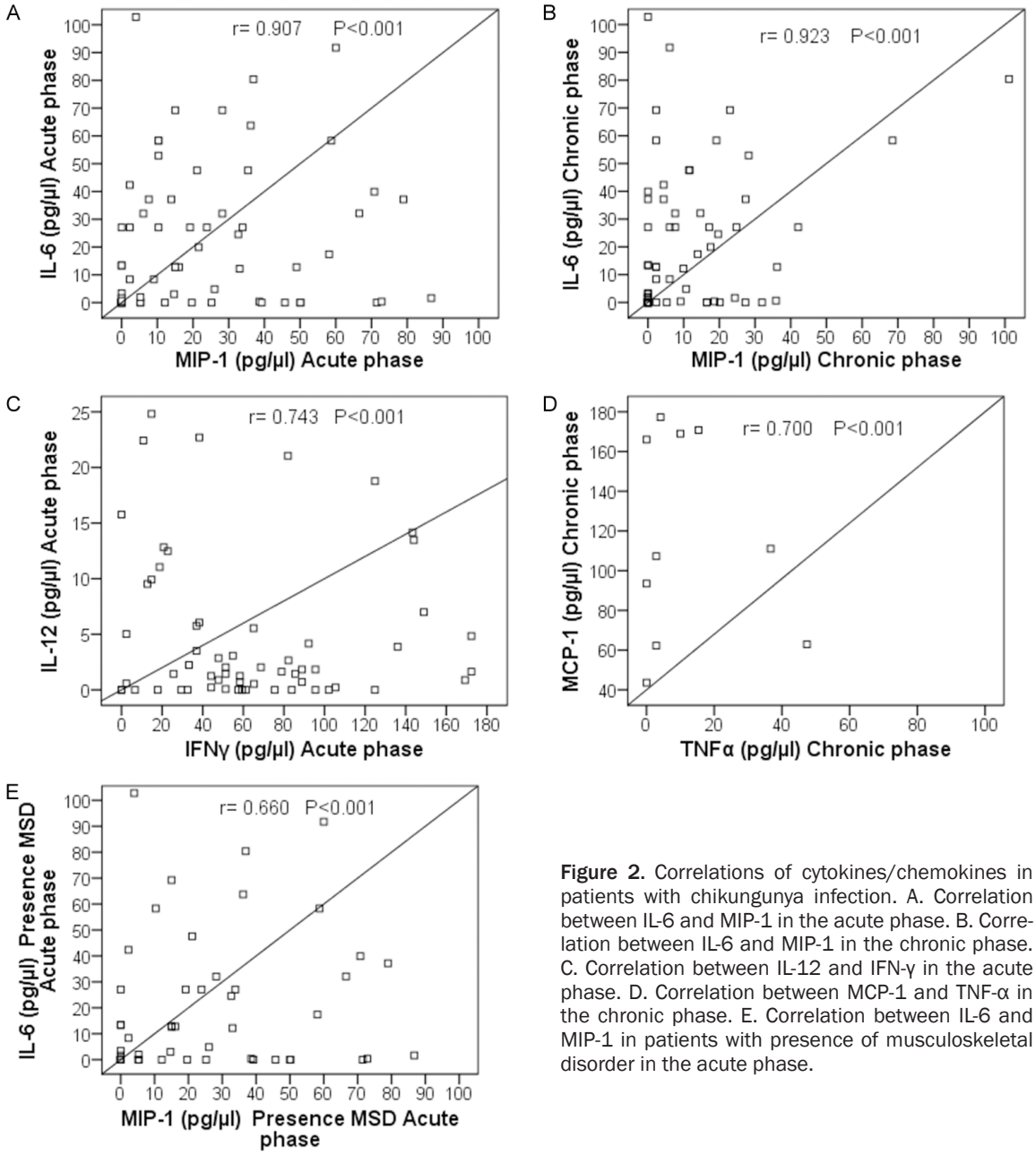


Figure 2. Correlations of cytokines/chemokines in patients with chikungunya infection. A. Correlation between IL-6 and MIP-1 in the acute phase. B. Correlation between IL-6 and MIP-1 in the chronic phase. C. Correlation between IL-12 and IFN-γ in the acute phase. D. Correlation between MCP-1 and TNF-α in the chronic phase. E. Correlation between IL-6 and MIP-1 in patients with presence of musculoskeletal disorder in the acute phase.

C3a, MCP-1, and RANTES/CCL5 were increased in patients with persistence of MSD. These results showed the predominant role of the chemokines in the development of MSD which is consistent with other studies [12, 14]. In the chronic phase, IFN-γ, IL-6, IL-12, IL-10, IL-8, and MIP-1 were more elevated in patients without the persistence of MSD, contrary to what was found by other authors [9, 10, 25]; nevertheless, in a meta-analysis, in spite of these last biomarkers and other having elevated levels in non-recovered patients, the differences were not significant [13]. This means that this topic might not be fully clarified.

According to severity of the MSD, we observed arthritis in 38.7% and 28.6% of the patients in acute and chronic phases, respectively. In line with the previously reported data, some authors reported arthritis in 20.5% of the patients in the acute phase, and around 7.4% to 25.2% in patients in the chronic phases [4, 18, 33], as opposed to another study that reported 53.7% of arthritis in the chronic phase [34]. This difference could be explained because, in this previous study, the population was constituted mostly of postmenopausal women. Alternatively, we found that severe joint pain occurred in 51.6% and 28.6% of the patients in the acute

and chronic phases, respectively, and severe functional disability in 21.0% and 3.6%, respectively, in both phases. Other authors had reported disability in 10.5% to 16.6% in the chronic phase [20, 35].

We observed higher C3a, IL-8, IL-10, and IL-12 levels in patients with arthritis and severe disability. On the other hand, elevated levels of MCP-1 were associated with arthritis and with severe disease. In contrast, RANTES levels were lower in patients with severe pain and severe disability. Other authors have associated the severity with increased levels of IFN-γ, IL-6, IL-12, interferon γ-induced protein 10 kDa (IP-10), IL-1b, monokine induced by interferon-gamma (MIG), MCP-1, IL-17A, IL-27 and reduced levels of RANTES, and IL-8 [13, 14, 36, 37]. The decrease in RANTES levels is a consequence of reducing platelets during the disease, which are its main reservoir [13, 29]. It is essential to highlight the role of MCP-1 in the induction of monocytes/macrophages, which are involved in the pathophysiology of arthritis [13].

In our study, MBL levels were significantly higher in the acute phase in comparison to the chronic stage; this could be explained because the role of MBL pathway in the complement activation in CHIKV infection. Previous studies have evidenced that activating the complement mediated by MBL pathway is essential in the severity progression of the infection RRV, another endemic arthritogenic alphavirus [15, 38].

As described above, we observed that elevated C3a levels are associated with chronicity and CHIKV infection severity. Studies have evidenced the role of C3a in enhancing the severity of RRV-induced disease in a murine model. Additionally, in patients with RRV polyarthritis, the levels of C3a in synovial fluid were significantly higher compared with patients with non-inflammatory osteoarthritis [39, 40].

In summary, our study showed the role of the complement system in CHIKV infection and an evident immunological response that persisted until the chronic phase. Chemokines such as C3a, MCP-1, and RANTES were related to the persistence of MSD. C3a, IL-12, and MCP-1 levels were significantly involved with the severity of chikungunya virus infection, suggesting they could be considered biomarkers of severity.

The recent findings about the mechanisms that CHIKV has developed to neutralize the complement system [41, 42] and our results highlight the importance of delving into the study of the role of the complement in this disease, as well as the need to identify biomarkers for patient follow-up and the development of possible treatments.

The limitation of our study was the low frequency of severe manifestations of MSD in the chronic phase, with the consequent decrease in the sample size in this patient group.

Finally, the differences in the frequency of persistence and severity of the CHIKV infection and the production of cytokines of the results between studies could be explained by the definition of variables, study population, and the measurement methods of immunology mediators.

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Disclosure of conflict of interest

None.

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