ARTHRITIS & RHEUMATOLOGY
Vol. 70, No. 4, April 2018, pp 585–593
DOI 10.1002/art.40383

Chikungunya Arthritis Mechanisms in the Americas

A Cross-Sectional Analysis of Chikungunya Arthritis Patients Twenty-Two Months After Infection Demonstrating No Detectable Viral Persistence in Synovial Fluid

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Objective. **To determine if chikungunya virus persists in synovial fluid after infection, potentially acting as a causative mechanism of persistent arthritis.**

Methods. **We conducted a cross-sectional study of 38 Colombian participants with clinical chikungunya virus infection during the 2014–2015 epidemic who reported chronic arthritis and 10 location-matched controls without chikungunya virus or arthritis. Prior chikungunya virus infection status was serologically confirmed, and the presence of synovial fluid chikungunya virus, viral RNA, and viral proteins was determined by viral culture, quantitative reverse transcription–polymerase chain reaction (qRT-PCR), and mass spectrometry, respectively. Biomarkers were assessed by multiplex analysis.**

Results. **Patients with serologically confirmed chikungunya arthritis (33 of 38 [87%]) were predominantly female (82%) and African Colombian (55%) or white Colombian (33%), with moderate disease activity (mean ± SD Disease Activity Score in 28 joints 4.52 ± 0.77) a median of 22 months after infection (interquartile range 21–23 months). Initial symptoms of chikungunya virus infection included joint pain (97%), swelling (97%), stiffness (91%), and fever (91%). The most commonly affected joints were the knees (87%), elbows (76%), wrists (75%), ankles (56%), fingers (56%), and toes (56%). Synovial fluid samples from all patients with chikungunya arthritis were negative for chikungunya virus on qRT-PCR, showed no viral proteins on mass spectrometry, and cultures were negative. Case and control plasma cytokine and chemokine concentrations did not differ significantly.**

Conclusion. **This is one of the largest observational studies involving analysis of the synovial fluid of chikungunya arthritis patients. Synovial fluid analysis revealed no detectable chikungunya virus. This finding suggests that chikungunya virus may cause arthritis through induction of potential host autoimmunity, suggesting a role for immunomodulating agents in the treatment of chikungunya**

ClinicalTrials.gov identifier: NCT02463968.

The views expressed herein are solely the responsibility of the authors and do not necessarily represent the official views of the Rheumatology Research Foundation, the NIH, or the US Army.

Supported by a Pilot award from the Rheumatology Research Foundation and the NIH (National Center for Advancing Translational Sciences grants UL1-TR-001876 and KL2-TR-001877).

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Submitted for publication July 14, 2017; accepted in revised form November 14, 2017.

arthritis, or that low-level viral persistence exists in synovial tissue only and is undetectable in synovial fluid.

Patients infected with chikungunya virus present with fever, headache, muscle pain, rash, and joint pain. Following resolution of acute chikungunya virus infection, chronic arthritis may develop, often lasting months to years (1,2). Chikungunya virus had previously been restricted to Africa, Asia, Europe, and the Indian and Pacific Ocean regions (3), but, in 2013, chikungunya virus was first described in the Americas and has now infected >1.5 million people in this region (1). Outbreaks of the Asian strain of chikungunya virus leave approximately one-third of patients with persistent joint pain 18 months after infection (4,5).

After transmission by an *Aedes aegypti* or *Aedes albopictus* mosquito bite, chikungunya virus undergoes local replication and then dissemination to lymphoid tissue (6). While viremia lasts only 5–12 days (7,8), a study in non-human primates demonstrated that chikungunya virus persists in the lymphoid organs, liver, joints, muscle, and macrophages for up to 3 months and that chikungunya virus RNA remains in the spleen, liver, and muscle for extended periods (9). In another study, chikungunya virus RNA was detected in the synovial tissue of a patient 18 months after infection (10). Similar findings of inflammatory macrophage infiltrates (11,12) and synovial viral RNA persistence (13) have been seen in polyarthritis caused by another alphavirus, Ross River virus. These findings have led to the hypothesis that chikungunya virus might persist in the joint in cases of chronic arthritis.

Chikungunya virus is known to evade neutralizing antibodies by residing within apoptotic blebs (14). While in the blebs, chikungunya virus can infect neighboring cells in vitro (15). Furthermore, in vitro chikungunya virus inhibits RNA kinase needed to make antiviral messenger RNA transcripts, thereby freezing host antiviral protein synthesis (16). Interleukin-6 (IL-6), monocyte chemotactic protein 1 (MCP-1), and interferon-α (IFNα) have been detected during the acute phase of infection, and elevated levels of analytes, including IL-6, MCP-1, IFNγ-inducible 10-kd protein (IP-10), IL-1 receptor α, eotaxin, IL-17, and granulocyte–macrophage colony-stimulating factor (GM-CSF), have further been associated with disease severity, chronic arthralgia, and/or viral load (17–19).

There is currently no standard treatment for chikungunya virus–associated arthritis (20). However, several small studies have demonstrated clinical benefit from treatment with antivirals such as ribavirin (21) and immunosuppressants such as methotrexate (22–24), hydroxychloroquine (22), etanercept (23), adalimumab (23), and sulfasalazine (24). Further characterization of chikungunya virus disease pathophysiology is needed to provide a rationale for large-scale randomized clinical trials to evaluate the effectiveness of these potential therapies. If persistent chikungunya virus infection is responsible for ongoing arthritis, immunocompromising disease-modifying agents may be improper and potentially dangerous treatments. Alternatively, if chikungunya virus does not persist in the joint, then evaluation of immunomodulating agents could be useful. The objective of the Chikungunya Arthritis Mechanisms in the Americas (CAMA) study was to determine if there was evidence of chikungunya virus in the synovial fluid of patients with chikungunya arthritis in order to understand disease pathogenesis and, perhaps, guide chikungunya arthritis therapy.

PATIENTS AND METHODS

Setting. Patients were recruited from the Atlántico and Bolívar Departments of Colombia. In September 2014, the first locally acquired chikungunya virus case was reported in the Bolívar Department. During the height of the epidemic, from 2014 to 2015, many suspected chikungunya virus cases were reported in the Departments of Atlántico (2,480 cases) and Bolívar (5,997 cases).

Inclusion criteria. Participants were Spanish-speaking adults ≥18 years old. Chronic chikungunya arthritis was defined as clinically or laboratory-confirmed diagnosis of chikungunya virus infection, with persistent arthritis or arthralgias. Arthritis and arthralgias included knee pain and swelling for at least 3 months after diagnosis of chikungunya virus infection as well as joint pain at the time of follow-up. As per the Colombian Institute of Health, a clinically confirmed case of chikungunya virus infection is defined as a fever of >38°C, severe joint pain or arthritis, and the acute onset of erythema multiforme with symptoms not explained by other medical conditions. In addition, these individuals must reside in or have visited a municipality where evidence of chikungunya virus transmission is present or have traveled within 30 km of confirmed viral circulation. No patients were excluded for prior arthritis; prior arthritis status was included in the analysis. All suspected chikungunya virus cases were laboratory confirmed for the purposes of this study. Healthy controls were defined as participants from the same region who reported no history of chikungunya virus infection and did not present with arthritis.

Exclusion criteria. Subjects were excluded if they reported a known bleeding disorder or were receiving anticoagulant medications. The study also excluded children, adults unable to give consent, prisoners, and pregnant women.

Recruitment. In 2014–2015, as part of a chikungunya virus surveillance study across the Atlántico and Bolívar Departments, 907 patients with clinically confirmed (n = 424) or laboratory-confirmed (n = 483) chikungunya virus infection were referred by their primary care providers from clinics located in Barranquilla, Atlántico; Sabanalarga, Atlántico; and San Juan Nepomuceno, Bolívar. Of these patients, 65 were randomly selected for eligibility screening, of whom 38 were eligible for study participation in the chronic arthritis group with current knee joint pain. Patients were not eligible if they did not have persistent

knee pain after chikungunya virus infection. Ten healthy controls were also recruited from among volunteers at the clinic in Barranquilla, Atlántico. Synovial fluid was not collected from healthy controls since it was not clinically indicated.

Ethics statement. The study protocol was approved by The George Washington University Institutional Review Board (IRB; protocol 041612), the Universidad El Bosque (UB 387-2015), and the US Army Medical Research Institute of Infectious Diseases Human Research Protections Office (FY15-32). Research on human subjects was conducted in compliance with Department of Defense, Federal, and State statutes and regulations relating to the protection of human subjects, and adheres to principles identified in the Belmont Report (1979). All data collection and research on human subjects for this publication were conducted under an IRB-approved protocol. All participants were adults and provided written informed consent during an in-person interview.

Primary outcome measure. We hypothesized that persistent active viral replication is responsible for chronic arthritis and joint pain. Therefore, the primary outcome measure of the present study protocol was the identification of the presence of chikungunya virus in synovial fluid. Attempts to find evidence of chikungunya virus in synovial fluid included viral culture, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) for chikungunya virus RNA, and mass spectrometry analysis for viral proteins.

Secondary outcome measures. As part of this study, we evaluated clinical outcomes, such as the effect on daily living and arthritis severity as measured by the Disease Activity Score in 28 joints (DAS28) (25), a validated rheumatoid arthritis (RA) assessment tool that is a composite score of the number of tender joints, number of swollen joints, global disease activity during the most recent week measured on a scale of 0–100, and C-reactive protein (CRP) level. This clinical outcomes questionnaire was administered to all of the participants in a face-to-face interview. Laboratory studies in these patients included testing for plasma CRP level, serum IgM rheumatoid factor (IgM-RF) antibody, IgG-RF antibody, anti–cyclic citrullinated peptide (anti-CCP) antibody, and selected cytokines and chemokines.

Sample collection. After obtaining of informed consent and administration of a questionnaire concerning the participant's demographic characteristics and symptom history, blood was obtained by venipuncture. An orthopedic surgeon performed an arthrocentesis for primary evaluation of the swollen knee joint with needle lavage, where 0–20 ml of saline was injected at the discretion of the clinician. Samples collected in this manner are referred to as "synovial fluid." Blood samples were collected into 8-ml CPT cell preparation tubes with sodium citrate (catalog no. 362761; Becton Dickinson), and synovial fluid was transferred to cell preparation tubes in an effort to isolate cells from the fluid.

Sample preparation. The blood samples were centrifuged at room temperature (18–25°C) in a horizontal rotor for 20 minutes at 1,500 relative centrifugal force. Plasma was removed from the blood collection tubes and frozen at −80°C until analyzed. Synovial fluid samples were similarly centrifuged and frozen for subsequent analysis. There was no visible cell pellet after centrifugation of the synovial fluid; therefore, supernatant and any present cells were stored as one specimen at −80°C.

Data management. All patients were assigned a unique patient identification number, which was used in the database and for labeling of patient samples. All patient data were free of personal identifiers and were stored in the REDCap database at The George Washington University.

Anti-chikungunya virus IgG and IgM. IgG and IgM levels in plasma were assayed using InBios CHIKjj Detect enzyme-linked immunosorbent assays (ELISAs) (CHKG-R and CHKM-R). These assays provide a qualitative evaluation of the presence or absence of anti-chikungunya virus IgG and IgM and include controls to calculate an immune status ratio. Plasma was diluted 1:100 in kit dilution buffer and tested in duplicate, according to the manufacturer's instructions.

RNA isolation and qRT-PCR. RNA isolation was attempted from 140 μl of plasma or synovial fluid using a Qiagen QIAamp Viral RNA Mini kit (catalog no. 52904). Control samples contained spiked RNA, which was isolated in parallel to ensure recovery and detection by qRT-PCR. Samples were run on an ABI 7500 HT system under the conditions listed below.

Both plasma and synovial fluid samples were evaluated using an RNA UltraSense One-Step Quantitative RT-PCR System. A standard curve was run in parallel with the samples, with duplicate evaluation of samples ranging from 1×10^7 to 1×10^2 copies/ml. The assay limit of detection is ~80 copies/ml. The primer and probe sequences were as follows: 5′-GGGCTATTCTCTAAACCGTTGGT-3′ (forward), 5′-CTCCCGGCCTATTATCCCAAT-3′ (reverse), and 5′-FAM-TCTGTGTATTACGCGGATAA-3′ MGB NFQ (probe). These primer and probe sequences (located in the peptidase C9 domain of the nonstructural polyprotein) were designed against chikungunya virus of Asian lineage, since the strains currently circulating in South America are of the Asian lineage. The cycling program was as follows: 50°C for 15 minutes, 95°C for 2 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

To confirm the results of the first assay, a second qRT-PCR assay was used to retest the synovial fluid samples with different primers that were specifically designed against isolates emerging from Colombia (GenBank accession nos. KX496989.1 and KT192707.1) and also target sequences in the peptidase C9 region of the nonstructural polyprotein. Samples were tested using a Power SYBR Green RNA-to-CT 1-Step Kit (catalog no. 4389986; ABI). The assay limit of detection is ~100 copies/ml. The primer sequences were 5′-GGCAGTGGTCCCAGATAATTCAAG-3′ (forward) and 5′-GCTGTCTAGATCCACCCCATACATG-3′ (reverse). The cycling program was as follows: 48°C for 30 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Synovial fluid culture. Vero cells were cultured in 12-well plates to 90% confluency. The culture media consisted of RPMI 1640, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% HEPES. Media were removed, and 500 μl of synovial fluid was added to each well and incubated for 1 hour. Where > 500 μl of synovial fluid was available, multiple cultures were established. As a positive control, chikungunya virus (strain 15661) was added to 2 wells, at ~10 plaque-forming units (PFU)/well and 1 PFU/well, with the aim of confirming detection of low levels of viremia in the samples. After 1 hour, 2.5 ml of complete media was added to each well, and the cells were incubated for 4 days (passage 1). On the fourth day, the supernatant was transferred to fresh Vero cells (90% confluency) in a 6-well plate (passage 2). An additional 3 ml of media was added, and the cells were cultured an additional 3 days. On the third day, a 140-μl aliquot of supernatant was collected for analysis. Then 3 ml of the supernatant was transferred again to fresh Vero cells (90% confluency) in a 6-well plate (passage 3). An additional 3 ml of media was

added, and the cells were cultured an additional 3 days. On the final day, a 140-μl aliquot of supernatant was collected for analysis. Remaining supernatant was then removed, and the cells were lysed in Buffer AVL (Qiagen). Buffer AVL was also added to the supernatant samples according to the manufacturer's instructions. The samples were heated at 56°C for 1 hour and removed from the biosafety level 3 laboratory, and the presence of viral nucleic acid was measured using PCR as described above.

Biomarker analysis. Levels of IgG-RF and IgM-RF were measured using an Inova Diagnostics Quanta Lite kit according to the manufacturer's instructions, using plasma samples instead of serum samples. Anti-CCP antibody levels were measured using an Inova Diagnostics Quanta Lite CCP3.1 IgG/IgA ELISA (catalog no. 704550) according to the manufacturer's instructions. Plasma samples were diluted 1:100 and quantified based on the assay standard curve. Multiplex assessment of a panel of cytokines and chemokines was conducted using a custom Meso Scale Discovery assay kit. Analytes included IFNα2a, CRP, IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, GM-CSF, IL-1α, IL-12/23p40, IL-15, IL-17A, eotaxin, macrophage inflammatory protein 1β (MIP-1β), IP-10, MIP-1α, and MCP-1. Samples were diluted according to the manufacturer's instructions for each analyte.

Mass spectrometry analysis. *Sample preparation.* Twenty-five microliters of each synovial fluid sample was added to 200 μl of Solution UT8 (8*M* urea) and processed by filter-assisted sample preparation (FASP) per the manufacturer's protocol. Briefly, proteins are bound to the FASP filter (catalog no. MRCF0R030; Millipore) in UT8 and alkylated in 55 m*M* iodoacetamide followed by digestion with 40 ng/μl trypsin/Lys-C (Promega) overnight at 37°C. Peptides were eluted in 50 m*M* NaCl and subsequently desalted using C18 spin columns (catalog no. 89870; Pierce) per the manufacturer's instructions. Eluted peptides were dried to completion. Digests were stored at −20°C until analyzed by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS).

LC-MS/MS analysis and protein search. Sample digests were resuspended in 20 μl of 0.1% formic acid and mixed briefly. Using a Dionex 3000 RSLCnano system (Thermo Scientific), 2.5 μl of each digest was injected onto a precolumn (a PepMap100 C18 column with a particle size of 5 μm, length of 5 mm, and internal diameter of 0.3 mm) housed in a 10-port Nano switching valve using a flow rate of 10 μl/minute. The loading solvent was 0.1% formic acid in high-performance liquid chromatography–grade water. The precolumn eluent was directed to waste. After 5 minutes, the switching valve changed to backflush the trapped peptides from the precolumn onto an EASY-Spray analytical column (15 cm × 75 μm) packed with PepMap C18 (with a particle size of 3 μm and a pore size of 100Å; Thermo Scientific). A 2–42% B gradient elution in 95 minutes was formed using pump A (0.1% formic acid) and pump B (85% acetonitrile in 0.1% formic acid) at a flow rate of 300 nl/minute. The column eluent was connected to an EASY-Spray nanospray source (Thermo Scientific) with an electrospray ionization voltage of 2.2 kV. An Orbitrap Elite mass spectrometer (Thermo Scientific) with an ion transfer tube temperature of 300°C and an S-lens setting of 50% was used to focus the peptides. Low-resolution rapid collision-induced dissociation (CID) MS/MS spectra were acquired with an automatic gain control of 1×10^4 ions and a maximum injection time of 50 msec. The isolation width for ms/ms CID fragmentation was set to 2 daltons. The normalized collision energy was 35% with a q value of 0.250. The dynamic exclusion duration was 30 seconds.

Searches were performed with Proteome Discoverer software version 2.1 (Thermo Scientific) using a human and chikungunya virus subset of the Swiss-Prot_2016_10_05 database. Variable modifications used were methyl (DE), acetyl (K), deamidated (NQ), oxidation (M), and carbamyl (K). Cysteine carbamidomethylation was specified as a constant modification. The false discovery rate was set at 0.1%. Mass tolerances were 10 parts per million for the MS1 scan and 200 ppm for all MS/MS scans. Search results were filtered such that only high-confidence/unambiguous peptide spectral matches were used.

Table 1. Baseline characteristics of the CAMA study participants*

	Serologically confirmed chikungunya arthritis cases			
	No history of arthritis (n = 25)	History of arthritis (n = 8)	Controls (n = 10)	*P* for trend
Age, mean ± SD	56.0 ± 10.0	59.6 ± 12.2	31.7 ± 7.8	<0.0001
Body mass index, mean ± SD	30.0 ± 4.5	27.1 ± 5.8	24.7 ± 5.3	0.03
Women	20 (80.0)	7 (87.5)	7 (70.0)	0.99
African Colombian ethnicity	13 (52.0)	5 (62.5)	5 (50.0)	0.90
White Colombian ethnicity	8 (32.0)	3 (37.5)	4 (40.0)	–
Education level of high school or less	23 (92.0)	8 (100.0)	0 (0.0)	<0.0001
Presence of comorbidities	9 (36.0)	6 (75.0)	0 (0.0)	0.0025
>3 comorbidities	0 (0.0)	1 (12.5)	0 (0.0)	0.19
Comorbidities				
Rheumatoid arthritis	0 (0.0)	1 (12.5)	0 (0.0)	0.19
Osteoarthritis	0 (0.0)	3 (37.5)	0 (0.0)	0.0045
Ischemic heart disease	0 (0.0)	0 (0.0)	0 (0.0)	NA
Chronic kidney disease	0 (0.0)	0 (0.0)	0 (0.0)	NA
Chronic pulmonary disease	0 (0.0)	0 (0.0)	0 (0.0)	NA
Diabetes	1 (4.0)	1 (12.5)	0 (0.0)	0.39
Hypertension	7 (28.0)	4 (50.0)	0 (0.0)	0.05
Depression	1 (4.0)	0 (0.0)	0 (0.0)	0.99

* Except where indicated otherwise, values are the number (%). CAMA = Chikungunya Arthritis Mechanisms in the Americas; NA = not applicable.

Table 2. Chikungunya infection–related symptoms in serologically confirmed chikungunya arthritis cases at initial presentation*

	All cases (n = 33)	No history of arthritis (n = 25)	History of arthritis (n = 8)
Chikungunya virus–related symptoms			
Joint tenderness	32 (97)	25 (100)	7 (88)
Joint swelling	32 (97)	25 (100)	7 (88)
Joint stiffness	30 (91)	22 (88)	8 (100)
Fever	30 (91)	23 (92)	7 (88)
Rash	29 (88)	22 (88)	7 (88)
Commonly initially affected joints†			
Knees	27 (87)	19 (83)	8 (100)
Elbow	25 (76)	19 (76)	6 (75)
Wrist	24 (75)	18 (75)	6 (75)
Fingers	18 (56)	14 (58)	4 (50)
Ankles	18 (56)	13 (57)	5 (63)
Toes	18 (56)	15 (63)	3 (38)
Hips	8 (26)	6 (26)	2 (25)

* Values are the number (%).
† Data were not available for all cases.

Statistical analysis. For univariate tests across diagnostic groups, the chi-square test or Fisher's exact test was used to compare categorical variables, analysis of variance was used for normally distributed continuous variables, and the Kruskal-Wallis test was used for skewed continuous variables. SAS (version 9.3) was used for data analysis. *P* values less than 0.05 were considered significant.

Given the sample size, there was suboptimal statistical power for some comparisons of secondary outcomes. For example, when comparing confirmed chikungunya virus cases with and those without prior arthritis (n = 33) to controls without chikungunya virus or arthritis (n = 10) with regard to categorical variables, using a 2-tailed chi-square test with an alpha of 0.05, power was >0.80 only for an effect size where the proportions that were positive were on the order of 40% versus 1%. However, the study had more robust power for detecting differences in continuous variables. For example, power was >0.80 for detecting a difference of mean ± SD 39 ± 10 versus 30 ± 10 (Cohen's $d = 0.9$) between patients with chikungunya virus and controls using a 2-tailed *t*-test.

RESULTS

Baseline characteristics of the subjects. Prior chikungunya infection was serologically confirmed in 33 (87%) of the 38 cases by IgM ELISA (in 1 [3%] of 33) and IgG ELISA (in 33 [100%]). Patients with confirmed chikungunya arthritis were predominantly female (27 [82%] of 33) and African Colombian (18 [55%] of 33) or white Colombian (11 [33%] of 33), with an education level of high school or less (31 [94%] of 33). Compared to healthy controls, the patients with chikungunya arthritis tended to be older, have less education, and have at least 1 comorbidity (Table 1). Participants with chikungunya arthritis with a history of prior arthritis were comparable to those with no history of arthritis in terms of age, sex, ethnicity, and education level. One patient with confirmed chikungunya virus exposure self-reported preexisting RA but was found to be negative for RF and anti-CCP antibody.

Chikungunya infection–related symptoms. Chikungunya arthritis patients were assessed a median of 22 months (interquartile range 21–23 months) after chikungunya virus infection. Initial symptoms of chikungunya virus infection included joint pain (97%), joint swelling (97%), joint stiffness (91%), fever (91%), and rash (88%) (Table 2); these symptoms were reported at the time of initial infection. The joints most commonly affected initially were knees (87%), elbows (76%), wrists (75%), fingers (56%), ankles (56%), and toes (56%). At follow-up, most participants reported that their arthritis had an effect on their activities of daily living (82%) (Table 3). However, patients were not necessarily experiencing a disease flare at the time of sample collection. Thirty-eight percent of the participants reported missing school or work during their initial infection. At the time of sample collection, participants had a mean ± SD of 5.5 ± 5.4 tender joints and 3.0 ± 2.8 swollen joints. The mean ± SD patient-reported global disease activity measure (on a

Table 3. Chikungunya infection–related effects assessed in serologically confirmed chikungunya arthritis cases at follow-up a median of 22 months after infection*

	All cases (n = 33)	No history of arthritis (n = 25)	History of arthritis (n = 8)	*P*
Effect on activities of daily living, no. (%)	27 (82)	19 (76)	8 (100)	0.30
Missed work/school, no. (%)†	12 (38)	7 (29)	5 (63)	0.12
Tender joint count	5.5 ± 5.4	5.4 ± 5.5	6.0 ± 5.2	0.0002‡
Swollen joint count	3.0 ± 2.8	2.8 ± 2.4	3.9 ± 4.0	<0.0001‡
Patient's global assessment of disease activity	93.4 ± 14.3	91.3 ± 15.9	100.0 ± 0	0.14
DAS28-CRP	4.52 ± 0.77	4.44 ± 0.73	4.78 ± 0.87	0.29

* Except where indicated otherwise, values are the mean ± SD. DAS28-CRP = Disease Activity Score in 28 joints using the C-reactive protein level.
† Data were not available for all cases.
‡ Using nonparametric Kruskal-Wallis test.

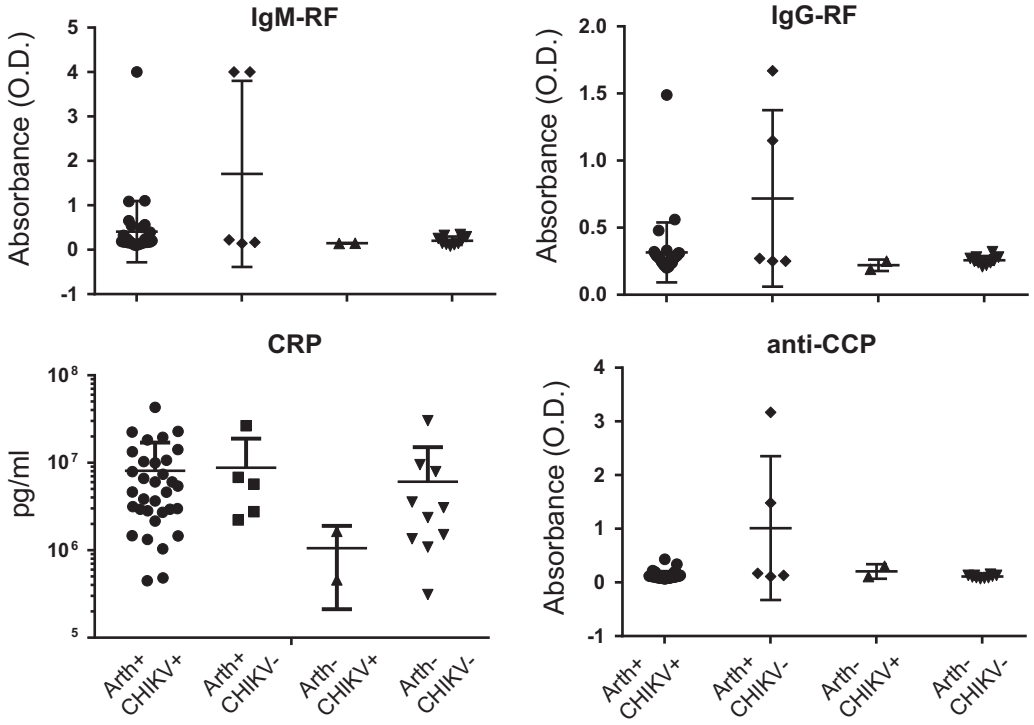

Figure 1. Levels of IgM rheumatoid factor (IgM-RF), IgG-RF, C-reactive protein (CRP), and anti–cyclic citrullinated peptide (anti-CCP) antibodies in patients with chikungunya virus (CHIKV) and arthraglia (Arth), patients with arthralgia without chikungunya virus, patients with chikungunya virus without arthralgia, and those without arthralgia or chikungunya virus. There was no significant increase in rheumatoid arthritis–associated markers or CRP level in patients with chikungunya virus–associated arthritis. Symbols represent individual subjects; horizontal lines and error bars show the mean ± SD.

scale of 0–100 with 100 being the most active) in the last week was 93 ± 14. The disease severity was moderate, as indicated by a mean ± SD DAS28 using the CRP level of 4.52 ± 0.77. There were no significant differences between chikungunya arthritis patients with a prior history of arthritis and those without a prior history of arthritis, with the exception of ~1 additional joint being tender and swollen in the participants with prior arthritis.

Virologic and serologic outcomes. All samples tested for persistent viral RNA (both plasma and synovial fluid) were negative for chikungunya virus on 2 separate qRT-PCR assays. To more rigorously evaluate if low-level viremia might be present in the samples, synovial fluid samples were added to cell cultures in an attempt to expand out any replication-competent virus. Cultures of synovial fluid from chikungunya arthritis patients also showed no viral growth after 3 passages and 10 days of culture; in contrast, controls with low quantities of virus (~1 PFU/well) yielded growth and allowed detection of the virus.

Plasma markers for RA were present in only a fraction of the participants with chikungunya virus–associated arthritis. IgM-RF antibody was present in 9% of the patients, and IgG-RF antibody was present in 12%. Anti-CCP antibody was not detected in any of the patients.

Table 4. Plasma cytokine and chemokine concentrations a median of 22 months after chikungunya infection*

Analyte	Chikungunya virus arthritis cases (n = 33)	Controls (n = 10)
Cytokine		
IL-1α	1.29 (0.58–3.82)	1.42 (0–4.93)
IL-1β	0.27 (0.17–0.69)	0.20 (0.10–0.28)
IL-2	1.70 (1.25–2.89)	2.06 (1.70–2.89)
IL-4	1.08 (0.76–2.80)	1.31 (0.61–2.51)
IL-6	1.69 (1.01–3.18)	1.32 (0.73–1.69)
IL-10	1.80 (1.29–2.56)	1.92 (1.29–2.08)
IL-12/p40	111 (92.0–137)	116 (87.4–161)
IL-12/p70	1.91 (1.37–6.20)	1.78 (1.05–6.79)
IL-15	2.55 (2.01–2.97)	2.64 (1.89–3.32)
IL-17α	7.81 (6.35–9.19)	7.2 (4.95–8.44)
IFNγ	7.44 (3.92–16.1)	6.7 (3.92–13.0)
GM-CSF	0.93 (0.60–1.30)	0.80 (0.32–1.15)
Chemokine		
Eotaxin	101 (68.0–136)	104 (65.0–134)
MIP-1α	23.9 (18.2–36.3)	27.6 (23.8–37.9)
IP-10	162 (123–227)	153 (133–197)
MCP-1	123 (88.3–140)	106 (87.5–144)
MIP-1β	46.6 (33.8–56.5)	38.13 (32.7–54.0)
IL-8	7.91 (6.15–16.9)	6.7 (5.44–11.4)

* Values are the median (interquartile range) pg/ml. IL-1α = interleukin-1α; IFNγ = interferon-γ; GM-CSF = granulocyte–macrophage colony-stimulating factor; MIP-1α = macrophage inflammatory protein 1α; IP-10 = interferon-γ–inducible 10-kd protein; MCP-1 = monocyte chemotactic protein 1.

Subjects with chikungunya virus–associated arthritis had no significant increase in RA-associated markers or CRP level (Figure 1). Interestingly, 2 of the 5 individuals who were enrolled in the study as having clinical chikungunya virus–associated arthralgia, but who were found to be seronegative for chikungunya virus, were positive for RF and/or anti-CCP antibody, suggesting they may have actually had RA or another related disease unlinked to chikungunya virus infection.

Plasma cytokine and chemokine data demonstrated trends that suggested differences between the patients with chikungunya virus with arthralgia and the controls, but which failed to reach statistical significance (Table 4). As observed in other studies (10,26,27), IL-6, IL-12p70, MCP-1, MIP-1β, and IL-8 were modestly elevated in patients compared to controls.

Proteomic analysis of synovial fluid. Mass spectrometry was used to identify proteins present in the synovial fluid of patients with arthralgia associated with chikungunya virus. The primary aim of the analysis was to determine if mass spectrometry could detect the presence of viral proteins, since this would suggest the persistence of antigen. However, mass spectrometry did not identify any chikungunya virus viral proteins in the fluid.

DISCUSSION

The CAMA study is the largest observational study involving synovial fluid analysis of patients with chikungunya arthritis in the Americas to date. We hypothesized that persistent active chikungunya virus is responsible for chronic arthritis and joint pain and that chikungunya virus viral RNA would be present in the synovial fluid. However, this study did not demonstrate viable virus after culture of synovial fluid in any of the participants who were studied, a median of 22 months after infection. Similarly, PCR analysis did not reveal viral RNA in the plasma or in the synovial fluid. Furthermore, proteomic analysis showed no evidence of viral proteins in the synovial fluid. These results have important implications for determining the mechanisms of persistent arthritis in patients with chikungunya virus and suggest that either there is no chikungunya virus in synovial fluid or that chikungunya virus does not replicate to high enough levels for detection in the synovial fluid 2 years after infection.

This study was inspired by the work of Hoarau et al (10), who found chikungunya virus antigen in macrophages and chikungunya virus RNA in synovial biopsy tissue 18 months after chikungunya virus infection in a single subject. In contrast, in our analysis of 33 patients (many with relapsing–remitting disease) 22 months after acute infection, we did not identify viral RNA or proteins, suggesting that viral persistence may not be a requirement for persistent joint pain. Another consideration is that synovial tissue analysis, as opposed to synovial fluid analysis, may permit improved viral recovery. Furthermore, this study may suggest that the pathophysiology behind human relapsing–remitting chikungunya virus–associated arthritis may differ from the pathophysiology of continuous erosive arthropathy seen in some patients.

In the context of animal studies, studies of nonhuman primates (9) and mice (28) have demonstrated viral persistence up to 44 and 100 days, respectively. Given our evaluation at significantly longer times after infection (22 month), it is conceivable that any virus present was eliminated by this later end point. Furthermore, murine models have yet to reproduce the relapsing–remitting nature of human chikungunya arthritis, suggesting that differing pathophysiology may be at play.

From a clinical perspective, patients with chikungunya virus–associated arthritis described substantial clinical disease burden. Eighty-two percent reported arthritis affecting their activities of daily living and moderate disease severity, as measured by the DAS28. This is consistent with other studies following up patients after chikungunya virus infection that have demonstrated symptoms of persistent arthralgia that may be relapsing or unremitting, often affect multiple joints, and are associated with functional loss impairing activities of daily living and reduced quality of life (29,30).

Multiple studies have shown elevated levels of inflammatory analytes during acute chikungunya virus infection, with IL-8, MCP-1, IL-6, MIP-1α, IL-1α, and MIP-1β reportedly elevated in some chronic chikungunya virus–associated arthralgia cohorts (10,26,27). We measured the levels of these cytokines and chemokines as well as several relevant RA-associated biomarkers. Consistent with the literature, RA-associated factors such as RF and anti-CCP were not elevated in our chikungunya virus–associated arthralgia cohort (2,23,31). Of interest, there were no significant differences in cytokine and chemokine levels within our cohort compared to location-matched controls at a median of 22 months. This might be the result of the very late stage of disease that we studied or the size of our control cohort. There was a trend toward elevation of proinflammatory markers (most notably IL-6) in our cohort, but whether a greater number of subjects would yield statistical significance can only be speculated.

It is possible that the apparent lack of a consistent pattern of markers of inflammation in patients with reported joint pain may be related to the relapsing–remitting nature of the joint pain described by patients with chikungunya arthritis. Patients describe periods of relative relief and “flare” periods of worsened joint symptoms in

response to physical stress or infection. Such relapse was also described by Borgherini et al in their study of the Réunion Island outbreak (32). In our study, while all patients reported joint pain, the relative intensity varied.

Given these results, additional potential mechanisms of the persistence of arthritis symptoms in the absence of chikungunya virus persistence in synovial fluid should be considered. First, it is possible that chikungunya virus or viral antigens persist at low levels in synovial tissue that cannot be detected in synovial fluid. Other potential mechanisms include chikungunya virus–induced epigenetic modifications of host DNA resulting in persistent alterations of host gene transcription, as has been seen in other viruses such as Epstein-Barr virus (33). Alternatively, macrophages could be modified through epigenetic imprinting, much like fibroblast-like synoviocytes are in RA, leading to more aggressive cell behavior even in the absence of replicating virus (34). Molecular mimicry may also play a role in chikungunya virus–induced arthritis, where the continued production of a chikungunya virus–specific antibody that cross-reacts with antigen in the synovium could account for chikungunya virus–associated inflammation. Finally, although unlikely, patients could have seronegative RA, or alternatively, seronegative RA could reflect prior infection with chikungunya virus or other arthritogenic viruses.

There are several limitations to this study that need to be addressed. First, during sample collection, 0–20 ml of saline was used to flush the joints, and this could affect our ability to detect virus in the synovial fluid samples. To mitigate this problem, we cultured 0.5–1.5 ml (as available) of the collected synovial fluid from each patient to attempt to expand any replication-competent virus in the samples. We also used 2 complementary PCR assays to detect nucleic acid as well as a proteomic approach to look for viral proteins. Proving the absence of a target is difficult, and we recognize that it is possible that our approach failed to detect low-level viral antigen; however, our orthogonal approach clearly demonstrates that if viral antigen exists in the synovial fluid, it is at extremely low levels. Though it is a more invasive procedure, future studies may benefit from using synovial biopsy rather than examining fluid. The advent of new ultrasound-guided biopsy techniques may permit this approach in the future. A second limitation of this study is the lack of control subjects who had a history of chikungunya virus infection without chronic arthritis, as well as the lack of age- and sex-matched healthy controls. Furthermore, our healthy controls were on average younger than the chikungunya virus–affected patients, and age is known to be associated with increased production of inflammatory cytokines, most notably IL-6 (35,36).

These study findings may have important clinical relevance for chikungunya virus in the Americas. Since there is no current standard of care guidance for treatment of chikungunya arthritis, some patients are currently being treated with immunosuppressive agents such as methotrexate (22–24), hydroxychloroquine (22), etanercept (23), adalimumab (23), sulfasalazine (24), fingolimod (37), abatacept, and tofacitinib (38). This practice could be potentially harmful in the setting of replicating virus in the synovium since it could permit re-emergence of a systemic viral infection. To date, no such resurgence has been reported. The failure to detect viral persistence in the synovial fluid in the present study may provide some reassurance that treatment with antirheumatic immunosuppressants 2 years after infection is a viable option.

ACKNOWLEDGMENTS

This study was made possible by the support of the following institutions and individuals: Allied Research Society, Falab Laboratory, La Universidad Metropolitiano, La Universidad El Bosque, The George Washington University, La Fundación Santa Fe, El Laboratorio Fals Borda, Lorena Encinales, Walberto Mariaga, Slyvana Insignares, Cesly Arteta, Martha Castillo Jimenez, Jaime Cantillo, April Barbour, Alan Wasserman, Donna Embersit, Sofia Tang, Armando Gonzalez Brunal, Olga Lucia Barran, Margarita Arroyave-Wessel, Fabiola Pomgrico, Julio Cesar Padilla, Jose Ziade, Beda Brichacek, Michael Bukrinksy, Pierre Roques, David Boyle, and Andreas Suhrbier.

ADDITIONAL DISCLOSURES

Author Cure is an employee of Biomelab.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Chang had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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