Chikungunya Arthritis Mechanisms in the Americas (CAMA):

A cross-sectional analysis of chikungunya arthritis patients 22-months post-infection demonstrates a lack of viral persistence in synovial fluid

SHORT TITLE: Chikungunya arthritis and synovial fluid viral persistence

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Abstract

Background. Chikungunya virus (CHIKV) is a mosquito-borne virus that causes chronic joint pain for months to years in approximately one half of infected patients. The study objective was to determine if CHIKV persists in the synovial fluid, potentially serving as a causative mechanism of persistent arthritis.

Methodology.

Design: Cross-sectional

Setting: Atlántico and Bolívar Departments, Colombia

Participants: Thirty-eight patients with CHIKV infection during the 2014-2015 epidemic with chronic arthritis including in the knee joint, and ten healthy controls without prior CHIKV infection were included.

Measures: Symptom questionnaire was performed. Prior infection with CHIKV was confirmed by serological analysis. The presence of chikungunya viral RNA in blood and synovial fluid were measured by qPCR.

Results. Prior CHIKV infection was serologically confirmed in 33/38 (87%) of the cases based on IgM (3%) and IgG ELISA (100%). Confirmed chikungunya arthritis patients were predominantly women (82%), Afro-Colombian (55%) or White-Colombian (33%) with high school or less level of education (94%). CHIKV arthritis patients (Median 22-months (IQR 21-23) post-CHIKV infection) had moderate disease activity (Disease Activity Score-28 of 4.52 ± 0.77). Initial symptoms of CHIKV infection included joint pain (97%), joint swelling (97%), joint stiffness (91%), fever (91%), and rash (88%). The most commonly affected joints during initial infection were knees (87%), elbows (76%), wrists (75%), fingers (56%), and toes (56%). Synovial fluid analysis of all CHIKV arthritis participants was CHIKV qPCR negative, showed no viral proteins by mass spectrophotometry, and was culture negative.

Conclusions. This is one of the largest observational studies involving synovial fluid analysis of chikungunya arthritis patients. Synovial fluid analysis revealed no evidence of CHIKV by qPCR, mass spectrometry, or culture. This suggests that immunomodulating medications may potentially play a role in the treatment of chikungunya arthritis and suggests a possible mechanism whereby CHIKV causes arthritis through induction of host autoimmune pathology.

Author Summary

Chikungunya virus (CHIKV) infection causes a persistent debilitating arthritis in one half of patients. The causal mechanism is unknown and there is no standard treatment for this condition. Immunomodulating medications used in rheumatoid arthritis are being tested in this patient population. However, such therapeutics could be dangerous if active virus is still present and the treatment interferes with immune-mediated control of infection. There have been case reports of chikungunya viral persistence by immunohistochemistry in synovial tissue in humans as long as 18 months post-acute infection with persistent arthritis. The current study evaluated if the virus could be detected in patients approximately two years after initial infection. Surprisingly, no virus was detected in the synovial fluid or peripheral blood of subjects despite persistent joint pain. These results indicate that persistent virus may be rare in the synovial fluid of patients with CHIKV-associated arthritis. The use of immunomodulating medications may potentially be a useful approach for the treatment of this condition. Furthermore, these results suggest that CHIKV may induce immune-mediated mechanisms of synovitis and that the continued presence of the virus is not necessary for persistent symptoms.

Introduction

Chikungunya virus (CHIKV) is transmitted by mosquitoes and sporadic epidemics occur regularly in areas with *Aedes aegpyti or Aedes albopictus* mosquitos. Infection presents with fever, headache, muscle pain, rash, and joint pain. Following resolution of the acute syndrome, chronic arthritis and arthralgias may develop which often lasts for months to years (1,2). Outbreaks had previously been restricted to Africa, Asia, Europe, and the Indian and Pacific Ocean regions (3). In 2013, CHIKV was described for the first time in the Americas in the Caribbean and has now infected over 1.5 million people (1). There is a risk that the virus will be imported to new areas outside the Caribbean basin by infected travelers.

Transmission of CHIKV occurs following a mosquito bite after which the virus undergoes local replication and dissemination to lymphoid tissue(4). Although viremia only lasts 5-12 days (5,6), a study in non-human primates has demonstrated that CHIKV persists in lymphoid organs, liver, joint, muscle, and macrophages up to 3 months after viral inoculation and that CHIKV RNA continues to replicate in spleen, liver, and muscle for extended periods (7). Furthermore, there is a case report of a human patient with persistent CHIKV RNA in the synovial tissue 18 months after infection (8). Within infected cells, CHIKV activates toll like receptors (4), stimulating an inflammatory cascade of cytokines such as interferon (IFN)-α, interleukin-6 (IL-6), and tumor necrosis factor (TNF) that may contribute to arthritis (9). These findings have led to the hypothesis that CHIKV might persist in the joint in cases of chronic arthritis.

There is currently no evidence-based standard treatment for CHIKV (10). However, several small studies have demonstrated clinical benefit with a variety of agents including ribavirin (11), methotrexate (12-14), hydroxychloroquine (12), etanercept (13), adalimumab (13), and sulfasalazine (14). Further characterization of the disease pathophysiology is needed to provide the rationale for large scale randomized therapeutic trials to evaluate the effectiveness of potential therapeutics. If persistent CHIKV infection is responsible for ongoing arthritis, immunocompromising disease modifying agents may be improper and potentially dangerous treatments. Alternatively, if CHIKV does not persist in the joint then evaluation of immunomodulating arthritis agents could be useful. The objective of the Study of Chikungunya Arthritis Mechanisms in the Americas

(CAMA) was to determine if there was evidence of CHIKV in the synovial fluid of patients with chikungunya arthritis in order to understand disease pathogenesis and, perhaps, guide chikungunya arthritis therapy.

Materials and Methods

Setting. Patients were recruited from the Atlántico and Bolívar Departments, Colombia. In Colombia, the first imported case of virus was confirmed using serological tests (IgM virus) in the laboratory of the National Institute of Virology Health on July 19, 2014. The case was a 71-year-old woman from the Dominican Republic who came to Colombia via the City of Palmira on July 15, 2014. On September 11, 2014, the first locally acquired case was reported in the village of San Joaquin, municipality of department Mahates, Bolivar. The Atlántico and Bolívar Departments border the Caribbean Sea. During the height of the epidemic from 2014-2015, many CHIKV cases were reported in the Departments of Atlántico (2,480 cases) and Bolivar (5,997 cases).

Inclusion criteria. Included participants were adults ≥18 years old, Spanish speaking, and able to give informed consent. Chronic chikungunya arthritis was defined as a clinical or laboratory confirmed diagnosis of CHIKV infection with persistent arthritis or arthralgias, including knee pain and swelling for at least three months after diagnosis of CHIKV infection. As per the Colombian Institute of Health a clinically confirmed case is defined as a patient presenting with fever greater than 38°C, severe joint pain or arthritis and acute onset of erythema multiforme with symptoms that are not explained by other medical conditions; residing or have visited a municipality where evidence of CHIKV circulation is present or is located in a municipality within 30 kilometers of confirmed viral circulation. All cases were then laboratory confirmed for the purposes of this study. Healthy controls were defined as participants from the same region who reported no history of prior CHIKV infection.

Exclusion criteria. Subjects were excluded if they reported a known bleeding disorder or if they were taking anticoagulant medications such as warfarin, clopidogrel, and ticagrelor therapy due to the increased bleeding risk. The study also excluded children, adults unable to give consent, prisoners, and pregnant women to prevent coercion of vulnerable populations.

Recruitment. In 2014-2015, as part of a CHIKV surveillance study across the Atlántico and Bolívar Departments 907 patients with a clinical (424) or laboratory (483) confirmed CHIKV infection were referred by their primary care providers from clinics located in Baranquilla, Atlántico; Sabanalarga, Atlántico; and San Juan Nepomuceno, Bolívar. From these patients, 65 patients were randomly selected for screening of which 38 patients were eligible for study participation in the chronic arthritis group. Patients were not eligible if they did not have persistent knee pain after CHIKV infection. Ten healthy controls were also recruited.

Consent and enrollment. A research assistant enrolled qualifying patients in the study during an in-person visit prior to the patient's visit with an orthopedist. During this visit, the study was explained in detail and informed consent was obtained. The study protocol was approved by the George Washington University Institutional Review Board (Protocol # 041612), the Universidad El Bosque (Protocol UB 387-2015), and the United States Army Research Institute of Infectious Disease Human Research Protections Office (Log #FY15-32). The study was also registered in the ClinicalTrials.gov registry.

Primary outcome. We hypothesized that persistent active viral replication is responsible for chronic arthritis and joint pain. Therefore, the primary outcome was the identification of the presence of CHIKV in the synovial fluid and/or joint lavage fluid. Attempts to find evidence of CHIKV in synovial fluid included viral culture in Vero cells, quantitative polymerase chain reaction (qPCR) for CHIKV RNA, and mass spectrophotometry analysis for viral proteins.

Secondary outcomes. Additionally, we evaluated clinical outcomes such as the effect on daily living and arthritis severity as measured by the Disease Activity Score (DAS-28) (15) which is a validated rheumatoid arthritis (RA) assessment tool that is a composite score of the number of tender joints, swollen joints, global disease activity in the last week measured from 0-100, and the C-reactive protein (CRP). This clinical outcomes questionnaire was administered to all the participants in a face-to-face interview. Laboratory studies in these patients included plasma C-reactive protein (CRP), serum rheumatoid factor IgM antibody, rheumatoid

factor IgG antibody, anti-cyclic citrullinated peptide (anti-CCP) antibody and selected cytokines and chemokines.

Sample Collection. Following informed consent and administration of the questionnaire concerning the participant's demographics 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.

***Sample Preparation*.** The blood samples were centrifuged at room temperature (18-25ºC) in a horizontal rotor for 20 minutes at 1500 relative centrifugal force (RCF), and the plasma was removed and frozen at -80ºC until analysis. Synovial fluid samples were similarly centrifuged and frozen for subsequent analysis. Cells were not observed in the synovial fluid, therefore only supernatant was collected.

Data management. All patients were assigned a unique patient identification number and all samples were labeled with only this number. All patient data was void of personal identifiers and was stored in REDCap database at George Washington University, which is a Health Insurance Portability and Accountability Act (HIPAA) complaint database.

Anti-CHIKV IgG and IgM. IgG and IgM levels were assayed using the InBios CHIKjj *Detect*™ Detect ELISA assays (CHKG-R and CHKM-R) as per the manufacturer's instructions, with the exception that plasma was evaluated instead of serum. These assays provide a qualitative evaluation of the presence or absence of anti-CHIKV IgG and IgM and provide controls to calculate an Immune Status Ratio (ISR). The assays also include acceptance and rejection criteria based on controls. Plasma was diluted 1:100 and tested in duplicate.

RNA isolation and qPCR. RNA isolation was attempted from 140 µl of plasma or synovial fluid using the QIAGEN QIAamp Viral RNA Mini Kit (Cat no. 52904). Control RNA was spiked into control samples and isolated in parallel to ensure recovery and detection by qPCR.

Both plasma and synovial fluid samples were evaluated using the RNA UltraSense One-Step Quantitative RT-PCR System. A standard curve was run in parallel with samples, with duplicate evaluation of samples ranging

from 1 x 10^7 to 1 x 10^2. Forward primer: GGGCTATTCTCTAAACCGTTGGT. Reverse primer: CTCCCGGCCTATTATCCCAAT. Probe: 5' FAM-TCTGTGTATTACGCGGATAA 3' MGBNFQ.

To confirm the results of the first assay, a second qPCR assay was used to re-test the joint lavage fluid samples using different primers. Samples were tested using Power SYBR One Step RNA to Ct (ABI Cat No. 4389986). Forward primer: GGCAGTGGTCCCAGATAATTCAAG. Reverse primer: GCTGTCTAGATCCACCCCATACATG.

Culture of Synovial Fluid. Vero cells were cultured in 12 well plates to 90% confluency. Media was removed and 500 µl of synovial fluid was added to each well and incubated for one hour. As a positive control, CHIKV (strain 15661) was added to two wells each at approximately 10 pfu/well and 1 pfu/well, with the aim of confirming detection of low levels of viremia in the samples. After one hour, 2.5 ml of complete media was added to each well and the cells were incubated for four days (passage 1). On the fourth day, the media 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 ul aliquot of supernatant was collected for analysis. Remaining supernatant was then removed and the cells were lysed in Qiagen buffer AVL. Buffer AVL was also added to the supernatant samples in accordance with the manufacturer's instructions. The samples were heated at 56C for one hour, removed from the BSL-3, and the presence of viral nucleic acid was measured using PCR as described above.

Biomarker analysis. Levels of IgG and IgM rheumatoid factor (RF) were measured using the Inova Diagnostics QUANTA Lite® assays in accordance with the manufacturer's instructions, with the exception that plasma was evaluated instead of serum. Anti-cyclic citrullinated peptide (anti-CCP) antibodies were measured using the Inova Diagnostics QUANTA Lite® CCP3.1 IgG/IgA ELISA (Cat no. 704550) in accordance with the manufacturer's instructions. Plasma samples were diluted 1:101 and quantified based on the assay standard curve. Multiplex assessment of a panel of cytokines, chemokines, and other acute biomarkers was conducted using a custom MesoScale Discovery (MSD) assay kit. Analytes included IFNalpha2α, CRP, IFNγ, IL1β, IL2,

IL4, IL6, IL8, IL10, IL12p70, GMCSF, IL1α, IL12/23p40, IL15, IL17A, eotaxin, MIP1 β, IP10, MIP1α, and MCP1. Samples were diluted in accordance with the manufacturer's instructions for each analyte.

Mass Spectrometry analysis. Sample Preparation: Twenty-five µL of each synovial fluid sample were added to 200 µL Solution UT8 (8 M Urea) and processed by Filter Assisted Sample Processing (FASP) per the manufacturers protocol. Briefly, proteins are bound to the FASP filter *(Millipore MRCF0R030)* in UT8 and alkylated in 55Mm iodoacetamide followed by digestion with 40 ng/µL Trypsin/Lys-C (Promega) overnight at 37°C. Peptides were eluted in 50 Mm NaCl and subsequently desalted using C18 spin columns (Pierce 89870) per manufacturer's instructions. Eluted peptides were dried to completion by speed vac. Digests were stored at -20°C until analyzed by LC MS/MS.

LC-MS/MS Analysis and Protein Search: Sample digests were re-suspended in 20 µL of 0.1% formic acid and mixed briefly. A Dionex 3000 RSLCnano system (Thermo Scientific) injected 2.5 µL of each digest onto a pre-column (C18 PepMap 100, 5 µm particle size, 5mm length x 0.3mm internal diameter) 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 HPLC grade water. The pre-column eluent was directed to waste. After 5 minutes, the switching valve changed to backflush the trapped peptides from the pre-column onto an Easy-Spray analytical column (15 cm x 75 µm) packed with PepMap C18, 3 µm particle size, 100A porosity particles (Thermo Scientific, Inc.). 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 300nL/minute. The column eluent was connected to an Easy-Spray nanospray source (Thermo Scientific) with an electrospray ionization voltage of 2.2kV. An Orbitrap Elite mass spectrometer (Thermo Scientific, Inc.) with an ion transfer tube temperature of 300^0C and an S-lens setting of 50% was used to focus the peptides into the mass spectrometer. A top 15 data dependent MS/MS method was used to detect and characterize the tryptic peptides. The top 15 most abundant ions were selected in a 400-1600 amu survey scan (120,000 resolution FWHM at m/z 400) with a full AGC target value of 1e6 ions and a maximum injection time of 200ms. Low-resolution rapid CID ms/ms spectra were acquired with an AGC of 1e4 ions and a maximum injection time of 50ms. 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 ProteomeDiscoverer 2.1 (Thermo Scientific) using a Human and CHIKV subset of the SwissProt_2016_10_05 database. Variable modifications used were Methyl (DE), Acetyl (K), Deamidated (NQ), and Oxidation (M) and Carbamyl (K). Cysteine carbamidomethylation was specified as a constant modification. The false discovery rate (FDR) was set at 0.1%. Mass tolerances were 10 ppm 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 (PSM) were used.

Statistical Analysis. For univariate tests across diagnostic groups, we used chi-square or Fishers Exact test to compare categorical variables, analysis of variance for normally distributed continuous variables, and the Kruskal-Wallis test for skewed continuous variables. SAS (version 9.3, Cary, NC) was used for data analysis, with $p<0.05$ considered significant.

Sample size and statistical power. We had sub-optimal statistical power for some comparisons of secondary outcomes due to small sample size. For example, in comparing CHIKV-confirmed cases with and without prior arthritis (n=33) to controls without CHIKV (n=10) on categorical variables, using 2-tailed chi-square with alpha=0.05, power was >0.80 only for an effect size where the proportions positive were on the order of 40% vs. 1%. We had better power for detecting differences in continuous variables. For example, power was >0.80 for detecting a difference of 39±10 versus 30±10 (Cohen's d = 0.9) between CHIKV cases & Control using a 2-tailed t-test.

Results

Baseline characteristics. Prior chikungunya infection was serologically confirmed in 33/38 (87%) of the cases by IgM 1/33 (3%) and IgG ELISA 33/33 (100%). Confirmed chikungunya arthritis patients were predominantly women 27/33 (82%), Afro-Colombian 18/33 (55%) or White-Colombian 11/33 (33%) with high school or less level of education 31/33 (94%). As compared to healthy controls, the patients with CHIKV arthritis tended to

be older, with lower education and have at least one comorbidity (Table 1). Participants with CHIKV arthritis with a history of prior arthritis compared to those with no history of arthritis prior to CHIKV infection were comparable in terms of age, gender, ethnicity, and education level. One patient with confirmed chikungunya exposure self-reported pre-existent RA but was found to be rheumatoid factor and anti-CCP antibody negative.

Table 1. Baseline characteristics of CAMA study participants

Characteristic	Serologically confirmed CHIK Arthritis Cases with no prior history of Arthritis (n=25)	Serologically confirmed CHIK Arthritis with Prior History of Arthritis (n= 8)	Controls without CHIKV infection or arthritis (n= 10)	p-value
Age at baseline, mean (sd)	56.0 (10.0)	59.6 (12.2)	31.7 (7.8)	<0.0001
Female Gender, n (%)	20/25 (80%)	7/8 (88%)	7/10 (78%)	0.99
Ethnicity, n (%)				
Afro-Colombian	13/25 (52%)	5/8 (63%)	5/10 (50%)	0.90
White-Colombian	8/25 (32%)	3/8 (38%)	4/10 (40%)	
Mean educational level- high school or less, n (%)	23/25 (92%)	8/8 (100%)	0 (0%)	<0.0001
Body Mass index, mean (sd)	30.0 (4.5)	27.1 (5.8)	24.7 (5.3)	0.03
Prior Comorbidities				
Presence of comorbidities, n (%)	9/25 (36%)	6/8 (75%)	0 (0%)	0.0025
>3 comorbidities, n (%)	0	1 (13%)	0	0.19
Comorbidity n(%)				
Rheumatoid arthritis	1 (4.0%)	0	0	0.99
Osteoarthritis	0	1 (12.5%)	0	0.19
Ischemic heart disease	0	3 (37.5%)	0	0.0045
Chronic kidney disease	0	0	0	NA
Chronic obstructive pulmonary disease	0	0	0	NA
Diabetes	1 (4.0%)	1 (12.5%)	0	0.39
Hypertension	7 (28.0%)	4 (50.0%)	0	0.05
Depression	1 (4.0%)	0	0	0.99

Chikungunya infection related symptoms. CHIKV arthritis patients were a median 22 (Interquartile range 21-23) months post CHIKV infection (Table 2). Initial symptoms of CHIKV infection included joint pain (97%), joint swelling (97%), joint stiffness (91%), fever (91%) and rash (88%). The most commonly affected initial joints were knees (87%), elbows (76%), wrists (75%), fingers (56%), ankles (56%) and toes (56%). Participants reported an effect on their activities of daily living from their arthritis (82%). Thirty-eight percent of participants reported missed school or work during their initial infection. At follow-up, participants had on average 5.5 ± 5.4

tender joints and 3.0 ± 2.8 swollen joints. Patient-reported global disease activity measure (scored from 0-100 with 100 being the most active) in the last week was 93 ± 14. The disease severity was moderate as shown by an average Disease Activity Score-28 using C-reactive protein (DAS-28) of 4.52 ± 0.77. There were no significant differences between CHIKV arthritis patient with or without a prior history of arthritis with the exception of a mean of approximately one additional joint tender and swollen in the participants with prior arthritis.

Table 2. Chikungunya infection related symptoms

Characteristic	Serologically confirmed CHIKV Arthritis cases (n=33)	Serologically confirmed CHIKV Arthritis Cases with no prior history of arthritis (n=25)	Serologically confirmed CHIKV Arthritis with prior history of arthritis (n= 8)	p-value
Duration of initial CHIKV symptoms in months *median (IQR)*	21.7 (20.7-22.7)	21.7 (20.7-22.7)	21.7 (13.8-22.2)	0.27 [A]
CHIKV related symptoms, *n(%)*				
Joint pain	32/33 (97%)	25/25 (100%)	7/8 (88%)	0.24
Joint swelling	32/33 (97%)	25/25 (100%)	7/8 (88%)	0.24
Joint stiffness	30/33 (91%)	22/25 (88%)	8/8 (100%)	0.56
Fever	30/33 (91%)	23/25 (92%)	7/8 (88%)	0.99
Rash	29/33 (88%)	22/25 (88%)	7/8 (88%)	0.99
Commonly affected initial joints, *n(%)*				
Knees	27/33 (87%)	19/25 (83%)	8/8 (100%)	0.55
Elbow	25/33 (76%)	19/25 (76%)	6/8 (75%)	0.99
Wrist	24/33 (75%)	18/25 (75%)	6/8 (75%)	0.99
Fingers	18/33 (56%)	14/25 (58%)	4/8 (50%)	0.70
Ankles	18/33 (56%)	13/25 (57%)	5/8 (63%)	0.99
Toes	18/33 (56%)	15/25 (63%)	3/8 (38%)	0.25
Hips	8/33 (26%)	6/25 (26%)	2/8 (25%)	0.99
Affect on activities of daily living *n (%yes)*	27/33 (82%)	19/25 (76%)	8 (100%)	0.30
Missed Work/ School *n (%yes)*	12/33 (38%)	7/25 (29%)	5 (63%)	0.12
Tender Joint Count *mean (sd)*	5.5 (5.4)	5.4 (5.5)	6.0 (5.2)	0.0002 [A]
Swollen Joint Count *mean(sd)*	3.0 (2.8)	2.8 (2.4)	3.9 (4.0)	<0.0001 [A]
Global disease	93.4 (14.3)	91.3 (15.9)	100.0 (0)	.14

measure *(0-100)* *mean (sd)*				
DAS-28 *mean (sd)*	4.52 ± 0.77	4.44 ± 0.73	4.78 ± 0.87	.29

[A] Using non-parametric Kruskal-Wallis test.

Virologic and Serologic Outcomes. None of the participants were qPCR positive for persistent viral RNA in the plasma or synovial fluid. Culture of synovial fluid from CHIKV arthritis patients also showed no viral growth, though inoculation of cells with very low (~1 pfu/well) quantities of virus yielded growth and detection of virus. Markers commonly seen in blood of RA patients were present in only a fraction of participants with CHIKV arthritis (rheumatoid factor (RF) IgM antibody (9%), RF IgG antibody (12%)) and no patients had anti-cyclic citrullinated peptide (anti-CCP) antibody. Subjects with CHIKV associated arthritis had no significant increase in RA associated markers or CRP (Figure 1). Interestingly, two of the five individuals who were enrolled in the study as having clinical CHIKV-associated arthralgia, but who were demonstrated to be seronegative for CHIKV, exhibited positive RF and/or anti-CCP antibody, suggesting they actually had RA or another related disease unlinked to CHIKV infection.

Plasma cytokine and chemokine data demonstrated trends that pointed to differences between the CHIKV subjects with arthralgia and the controls, but differences did not reach statistically significance (Table 3). As observed in other publications, IL6, IL12p70, MCP-1, MIP-1B and IL8 were modestly, but not significantly elevated in convalescent subjects compared to controls. The failure to achieve significance may be due to the length of time since primary infection or may be due to the sample size of the control group: of the ten control individuals, three had apparently elevated inflammatory analytes, the origin of which is unknown.

Table 3. Plasma cytokine and chemokine concentrations.

Analyte	Confirmed CHIKV history (n=33) (pg/ml)	Controls (n=10) (pg/ml)	p-value
Type 1 Cytokines			
Interleukin-2 (IL-2)	1.70 (1.25-2.89)	2.06 (1.70-2.89)	0.12
Interleukin-4 (IL-4)	1.08 (0.76-2.80)	1.31 (0.61-2.51)	0.80
Interleukin-12 (IL-12-p40)	111 (92.0-137)	116 (87.4-161)	0.55
Interleukin-15 (IL-15)	2.55 (2.01-2.97)	2.64 (1.89-3.32)	0.63
Interleukin-17α (IL-17a)	7.81 (6.35-9.19)	7.2 (4.95-8.44)	0.41
Granulocyte-Monocyte	0.93 (0.60-1.30)	0.80 (0.32-1.15)	0.55

CSF (GM_CSF)			
Interleukin-6 (IL-6)	1.69 (1.01-3.18)	1.32 (0.73-1.69)	0.09
Interleukin-12 (IL-12-p70)	1.91 (1.37-6.20)	1.78 (1.05-6.79)	0.81
Type 2 Cytokines			
Interleukin-10 (IL-10)	1.80 (1.29-2.56)	1.92 (1.29-2.08)	0.85
Interferon-γ (IFN-γ)	7.44 (3.92-16.1)	6.7 (3.92-13.0)	0.82
IL-1 Superfamily Cytokines			
Interleukin-1α (IL-1α)	1.29 (0.58-3.82)	1.42 (0-4.93)	0.39
Interleukin-1β (IL-1β)	0.27 (0.17-0.69)	0.20 (0.10-0.28)	0.10
Chemokines			
Eotaxin	101 (68.0-136)	104 (65.0-134)	0.93
Macrophage inhibitory protein (MIP-1α)	23.9 (18.2-36.3)	27.6 (23.8-37.9)	0.22
Interferon-γ induced Protein (IP-10)	162 (123-227)	153 (133-197)	0.67
Monocyte chemotactic protein-1 (MCP-1)	123 (88.3-140)	106 (87.5-144)	0.75
Macrophage inhibitory protein (MIP-1β)	46.6 (33.8-56.5)	38.13 (32.7-54.0)	0.40
Interleukin-8 (IL-8)	7.91 (6.15-16.9)	6.7 (5.44-11.4)	0.12

***Proteomic analysis of synovial fluid*.** Mass spectrometry (MS) was used to identify proteins present in the synovial fluid of patients with arthralgia associated with CHIKV. MS did not identify any CHIKV viral proteins in the fluid. Supplemental Figure 1 shows a list of the most abundant proteins that were detected in the synovial fluid samples.

Discussion.

Potential mechanisms of persistent CHIKV arthritis. CAMA is the largest observational study involving synovial fluid analysis of patients with CHIKV arthritis in the Americas to date. We hypothesized that persistent active CHIKV is responsible for chronic arthritis and joint pain and that CHIKV viral RNA would be present in the synovial fluid. However, CAMA demonstrated a lack of viral RNA in the plasma as well as the synovial fluid by PCR and by detection of live virus after culture of the synovial fluid of all participants with CHIKV arthritis at a median 22 months post-infection. This finding was confirmed by proteomic analysis, which found no evidence of viral proteins in the synovial fluid. These results have important implications for determining mechanisms of the persistent arthritis in CHIKV patients and suggest that either there is no virus in synovial fluid or that CHIKV does not replicate at high enough levels for detection in the synovial fluid 2-years post-infection despite persistent evidence of synovitis. However, further studies of synovial tissue at earlier time

337 points post-infection may demonstrate evidence of low level replicating viral persistence or persistent viral
338 antigen as shown by Hoarau et al. (8) . In that study, persistent CHIKV RNA in perivascular macrophages was
339 described at 18-months post CHIKV infection in a single subject.
340
341 Multiple studies have found an elevation in inflammatory analytes during acute CHIKV infection, and IL8,
342 MCP1, IL6, MIP1α, IL1α, and MIP1β have been reported in some chronic CHIKV arthralgia cohorts (8,16,17) .
343 We measured these cytokines and chemokines as well as several relevant RA-associated biomarkers.
344 Consistent with the literature, RA-associated factors like RF and anti-CCP were not elevated in our CHIKV-
345 associated arthralgia cohort (2,13,18) . Of interest, we did not find significant differences in cytokine and
346 chemokine levels within our cohort compared with control. This might be the result of the very late stage of
347 disease that we studied or the size of our control cohort. However, this is also the first study looking at these
348 analytes in cohorts in the Americas, and there may be population differences that account for our failure to
349 detect such differences.
350
351 Given these results, additional potential mechanisms for the persistence of arthritis symptoms in the absence
352 of CHIKV persistence should be considered. Such potential mechanisms include CHIKV-induced epigenetic
353 modifications of host DNA resulting in persistent alterations of host gene transcription, as has been seen in
354 other viruses such as Epstein Barr Virus (19) . Alternatively, macrophages could be modified through
355 epigenetic imprinting, much like fibroblast-like synoviocytes are in RA leading to more aggressive cell behavior
356 (20). A role for molecular mimicry has also been proposed for CHIKV as it has for other diseases, including
357 EBV; the continued production of a CHIKV-specific antibody that cross-reacts with antigen in the synovium
358 could account for CHIKV-associated inflammation. Finally, although unlikely, patients could have seronegative
359 RA; alternatively seronegative RA could reflect prior infection with CHIKV or other arthritogenic viruses.
360
361 ***Clinical disease burden.*** Clinical disease burden was assessed as a secondary outcome with CHIKV arthritis
362 participants. Eighty-two percent reported arthritis affecting their daily living and a moderate disease severity, as
363 measured by the Disease Activity Score-28. This is consistent with other studies following patients after CHIKV
364 infection, which 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 (21,22) .

Clinical Relevance. There is no current standard of care guidance for treatment of CHIKV arthritis. However, some patients are being treated with immunosuppressant medications such as methotrexate (12-14), hydroxychloroquine (12), etanercept (13), adalimumab (13), sulfasalazine (14), fingolimod (23) , abatacept and tofacitinib (24) . Although this practice could be potentially harmful in the setting of replicating virus in the synovium, as it would permit the re-emergence of systemic viral infection, resurgence of systemic viral infection after immunomodulating medication treatment for CHIKV arthritis has not be reported. The findings from this study of lack of viral persistence detected in the synovial fluid provide some support that it may be safe to treat with immunosuppressant anti-rheumatic medications at two years post-infection.

Limitations. There are limitations of using synovial fluid for evaluation of arthritis. During collection, saline was used to flush the joints and this could affect the concentration of any virus in the synovial fluid samples. To adjust for this problem, we cultured 0.5 ml – 1.0 ml (as available) volume of the collected synovial fluid from each patient as well as running two PCR assays, increasing our confidence that we did not miss any circulating virus in the samples. Synovial biopsy might yield more information than synovial fluid in terms of biomarkers, but it is a more invasive procedure. The advent of new ultrasound guided biopsy techniques could permit this approach in the future.

Disclosure. The authors report no conflicts of interest in this work.

Disclaimer. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Research on human subjects was conducted in compliance with DoD, 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 and human subjects research were gathered and conducted for this publication under an IRB approved protocol, number FY15-32 .

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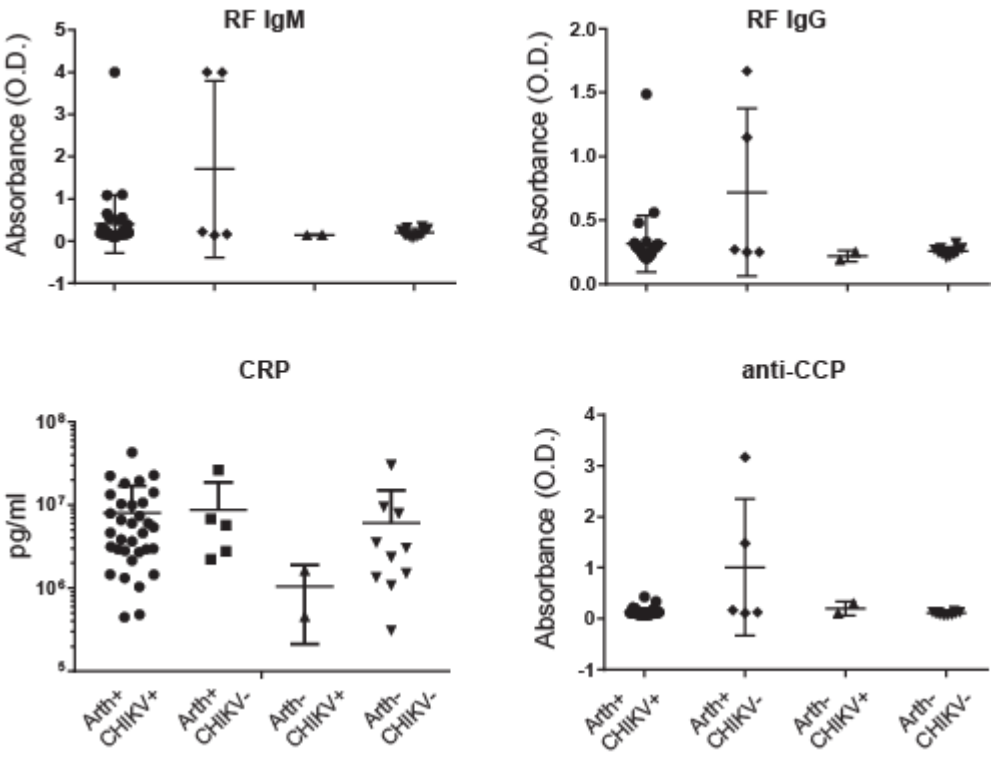
Figure 1. Subjects with CHIKV-associated arthritis had no significant increase in RA associated markers or CRP. Subjects were divided into four groups based on whether they presented with arthralgia or not (Arth+/-) and on whether they were IgG or IgM positive for anti-CHIKV antibody (CHIKV+/-).



Supplemental Appendix Table 1. Most commonly identified proteins found in synovial fluid by mass spectrophotometry in patients with chikungunya arthritis.

Accession #	Protein	Molecular Function
P01009-1	Alpha-1-antitrypsin	Protease inhibitor
P01023	Alpha-2-macroglobulin	Protease inhibitor
P02647	Apolipoprotein A-I	Lipase inhibitor
P04114	Apolipoprotein B-100	Lipase inhibitor
P00915	Carbonic anhydrase 1	Arylesterase activity
P00450	Ceruloplasmin	Ferroxidase activity
P01024	Complement C3	Endopeptidase inhibitor
P02751	Fibronectin	Peptidase activator
P00738	Haptoglobin	Antioxidant activity