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Transcriptomic insights into early mechanisms underlying post-chikungunya chronic inflammatory joint disease

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Chikungunya virus (CHIKV) infection often results in a chronic joint condition known as Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD). This condition disrupts individuals' daily lives and contributes to increased healthcare expenditure. This study investigated the molecular mechanisms underlying pCHIKV-CIJD development by analyzing RNA transcripts, including small RNAs, of whole blood from CHIKV-infected patients. By comparing patients who evolved to pCHIKV-CIJD with those who did not, we identified molecular signatures associated with chronification in acute and post-acute disease phases. These molecules were primarily associated with an altered immune response regulation. Notably, *LIFR*, an immune receptor that enhanced *IL-6* transcription, was down-regulated in the acute phase of pCHIKV-CIJD patients, while its inhibitor, hsa-miR-98-5p, was up-regulated in these individuals. Other downregulated genes include members of immune mechanisms whose impairment can lead to a reduction in the first line of antiviral response, thereby promoting virus persistence for a longer period in these patients. Additionally, pCHIKV-CIJD patients exhibited reduced transcript levels of *MMP8*, *LFT*, and *DDIT4*, genes already implicated in the pathological process of other types of inflammatory arthritis and seemingly relevant for pCHIKV-CIJD development. Overall, our findings provide insights into the early molecular mechanisms involved in the chronification and highlight potential targets for further investigation.

Keywords CHIKV, Chikungunya, Post-Chikungunya Chronic Inflammatory Joint Disease, Total RNA sequencing, Small RNA sequencing, Chikungunya prognosis

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CHIKV, the Chikungunya virus, was first isolated in 1952 during an epidemic of febrile illness that resembled dengue[1]. Autochthonous transmission of CHIKV was later confirmed in Brazil in 2014, and the country experiences the highest number of Chikungunya fever cases in the Americas[2]. The disease is characterized by fever, joint pain, headache, myalgia, and skin rash. While most patients recover within a week, some experience complications, including chronic musculoskeletal symptoms that can persist for months or even years[3].

Chikungunya fever is clinically divided into acute, post-acute, and chronic phases. The acute phase, lasting up to 14 days, is marked by symptoms such as fever, polyarthralgia, rash, and retro-ocular pain. The post-acute phase, from 14 to 90 days, is characterized by the persistence or recurrence of acute phase symptoms, with joint involvement including post-acute hypertrophic tenosynovitis. The chronic phase extends beyond 90 days and is associated with long-lasting arthralgia, inflammatory signs, and varying levels of severity. This condition, known as Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD) is the main determinant of morbidity in affected individuals[4,5].

In a recent study, we observed, through a longitudinal CHIKV cohort, that symptomatic post-acute disease is a relevant predictor of evolution to chronic arthritis with synovitis, drawing attention to joint pain, edema, and multiple articular involvements including small hand and feet joints as risk factors for chronification beyond three months, especially in women[5].

Transcritptomic analysis elucidated the immune related pathways disregulated during the CHIKV infection. In the CHIKV mouse model mostup-regulated genes are Type-I interferon response genes. In the same work the granzyme A was identified as an important driver of arthritic inflammation[6]. Corroborating this data, whole blood RNA-seq analysis of pediatric patients also found strong up-regulation of interferon-associated genes, such as IFIs, *MX1* and *MX2*, *OAS* genes, and *RSAD2*. Major histocompatibility complex transcripts were also associated with the disease's severity, where *HLA-B* transcripts were more expressed in patients with less severe acute-phase symptoms[7]. By comparing peripheral blood samples from CHIKV patients to those from healthy controls, a 2019 study identified transcriptional changes associated with CHIKV infection. Specific gene signatures, particularly those involving Eukaryotic Initiation Factor (eIF) family genes and *APOBEC3A*, were found to play significant roles in the viral replication process. Additionally, the study revealed that CHIKV infection activates the *NLRP3* inflammasome in macrophages, leading to the production of IL-1 beta, a key component of the immune response[8].

Advancements in understanding CHIKV infection have primarily focused on the acute phase, with limited insights into the molecular mechanisms driving the transition to chronic arthralgia. To address this knowledge gap, we conducted an in-depth analysis using advanced sequencing techniques to elucidate the molecular mechanisms underlying the development of pCHIKV-CIJD. Our study involved the examination of RNA transcripts, including both small and long non-coding RNAs, from whole blood samples collected from patients in a Brazilian cohort. We compared samples from individuals who progressed to pCHIKV-CIJD with those who did not, over a 90-day follow-up period. Our results revealed a significant downregulation of *LIFR*, an immune receptor implicated in *IL-6* transcription, alongside upregulation of its inhibitory microRNA hsa-miR-98-5p in patients who developed chronic disease.

Additionally, we observed a reduction in the expression of genes crucial for immune responses, which may contribute to diminished antiviral activity and prolonged viral persistence. Notably, reduced transcript levels of *MMP8*, *LTF*, and *DDIT4* were found in patients with pCHIKV-CIJD, highlighting their potential involvement in the pathogenesis of chronic inflammatory arthritis. These findings enhance our understanding of the molecular pathways involved in CHIKV chronification and suggest potential targets for therapeutic intervention.

Results and discussion

Total RNA analysis reveals distinct gene expression profiles early associated to chronification

By comparing patients who progressed to Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD) with those who did not, we comprehensively analyzed their transcriptomes in acute and post-acute phases. First, Principal Component Analysis (PCA) revealed that the main differences in the dataset are driven by the infection phase rather than group classification (pCHIKV-CIJD vs. non-pCHIKV-CIJD) (Figure S1). PC2 (24.28% variance) clearly separates the samples between the acute and post-acute phases, with acute-phase samples clustering predominantly lower on the axis and post-acute-phase samples higher. In contrast, PC1 (39.51% variance) captures additional variability but does not distinctly separate the groups. When performing the differential expression analysis we observed differences in gene expression profiles between the two groups. In the acute disease phase, we identified 83 differentially expressed genes (DEGs; Log2FoldChange $\geq |0.5|$ and FDR < 0.1), with 35 genes up-regulated and 48 genes down-regulated in patients who progressed to pCHIKV-CIJD (Fig. 1A, Table S1a).

In contrast, during the post-acute phase, the transcriptomic analysis revealed an increase in DEGs, with 458 genes showing differential expression. Among these, 427 genes were up-regulated and 31 were down-regulated (Fig. 1B, Table S1a). This substantial increase suggests a sustained and possibly intensified immune response in patients progressing to pCHIKV-CIJD, which could contribute to the persistence and exacerbation of inflammation and joint symptoms characteristic of chronic disease. Additionally, in a previous study conducted by our group with the same cohort of patients, it was observed that those who progressed to pCHIKV-CIJD exhibited more symptoms during the subacute phase of the disease, particularly signs of arthritis and joint pain. This observation was considered a risk factor for chronification[5]. This clinical correlation underscores the importance of our current molecular findings, as the increased symptomatology in the subacute phase may reflect the underlying molecular disruptions we have identified.

Since the acute phase, patients who evolved to pCHIKV-CIJD presented many differentially expressed long non-coding RNAs (lncRNAs), representing 20.5% (17/83) of the set of DEGs in this stage. Interestingly, this number reaches 65.1% (298/458) in the post-acute phase of the disease (Fig. 1C,D). LncRNAs are more than

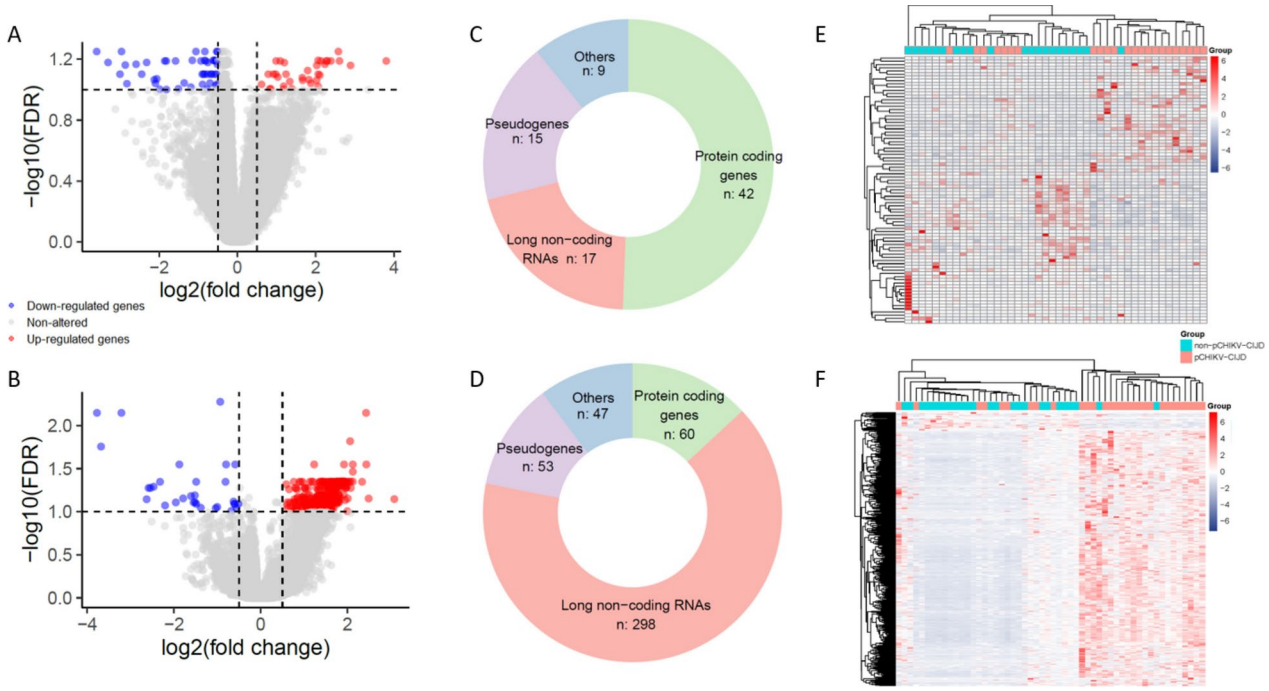

Fig. 1. Differences in gene expression between patients who evolve or not to pCHIKV-CIJD in different phases of CHIKV infection. (**A**, **B**) Volcano Plot representing the differentially expressed genes (DEGs) found in the comparison between patients pCHIKV-CIJD and non-pCHIKV-CIJD in the acute phase (**A**) and post-acute phase (**B**) of infection. Points on the plot represent genes, with the x-axis showing the log2FoldChange and the y-axis showing the -log10 of the FDR-adjusted p-value. Genes significantly regulated are highlighted in red (up-regulated) or blue (down-regulated), while genes not significantly regulated are shown in gray. (**C**, **D**) Classification of the DEGs found in patients pCHIKV-CIJD compared to those non-pCHIKV-CIJD in the acute phase (**C**) and post-acute phase (**D**) of infection. The DEGs are categorized into different functional classes based on their known or predicted biological function. (**E**, **F**) Hierarchical clustering heatmap depicting the global expression profile of DEGs between samples from patients pCHIKV-CIJD and non-pCHIKV-CIJD in the acute (**E**) and post-acute (**F**) phases of infection. The rows represent genes, and the columns represent samples. The color shades indicate gene expression levels, with lighter shades indicating lower expression and darker shades indicating higher expression.

200 bp and represent the majority of non-coding RNAs in humans with 127,802 lncRNA transcripts sequenced and annotated[9] have been increasingly associated with the control of many cell functions[10] by several different mechanisms, such as regulation of gene transcription[11]; altering the standard function of proteins[12]; function as competing endogenous RNA (ceRNA) being able to bind to multiple microRNAs, which regulate their targets expression[13]; and participating in the antiviral immune response, directly and indirectly controlling the production of anti-viral molecules in addition to pro-inflammatory cytokines[14] and interferons[15]. The significant presence of lncRNAs among the DEGs found here, particularly in the post-acute phase, highlights their potential regulatory roles in the pathological processes leading to pCHIKV-CIJD.

Despite the number of studies increasing in recent years, there are still few lncRNA with a well-described function and more studies are needed to evaluate its role in the response to CHIKV infection and its progression. In 2022, Basavappa et al. employed RNAseq to analyze gene expression in HBMECs infected with CHIKV and ZIKV, discovering that a substantial portion of differentially expressed genes were lncRNAs, many of which were virus-specific. These findings highlight the pivotal, virus-dependent role of lncRNAs in the antiviral response. Among these lncRNAs, the authors detected the overexpression of ALPHA in response to CHIKV infection. They showed that ALPHA overexpression decreases CHIKV replication by interacting with viral RNA and preventing its translation, thus reducing viral protein production. Additionally, ALPHA enhances the host's immune response by promoting interferon-stimulated genes, which is crucial for antiviral defense against chikungunya virus[16].

Here, we did not find differences in ALPHA expression in the pCHIKV-CIJD group that could explain increased viral persistence in these patients in the acute and post-acute phases. However, the high number of lncRNAs in the post-acute phase suggests they play a crucial role in regulating of immune and inflammatory responses, potentially influencing the progression to chronic disease. Although the role these lncRNAs play in these clinical conditions remains to be elucidated, our interactome analyses showed that some differentially expressed lncRNAs interacted with genes found in the enrichment core of altered pathways in post-acute phase, as Neutrophil degranulation and Class I MHC mediated antigen processing and presentation (Table S2).

To further understand gene expression patterns, we performed hierarchical DEGs clustering in the acute (Fig. 1E) and post-acute (Fig. 1F) phases. This analysis demonstrated a clustering of patients according to their prognosis, with those progressing to pCHIKV-CIJD showing distinct gene expression profiles. This consistency in gene expression patterns within patient groups underscores the molecular differences between those who develop chronic disease and those who do not.

Gene Set Enrichment Analysis (GSEA) is a computational method used to analyze gene expression data, focusing on differences in expression and statistical significance. This method identifies coordinated changes in predefined gene sets related to biological pathways or processes, providing insights into the functional significance of molecular changes in disease states. GSEA allows us to determine if a particular class of genes is over-represented in a condition, in our case, pCHIKV-CIJD.

Figure 2 shows the GSEA results for expressed genes, highlighting differences in expression and statistical significance to identify enriched pathways associated with Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD) across acute and post-acute phases. The top ten pathways, selected based on statistical significance and negative Normalized Enrichment Scores (NES), indicate a predominant down-regulation of core genes in pCHIKV-CIJD patients compared to non-pCHIKV-CIJD individuals (detailed statistics are in Table S3).

Through GSEA analyses, we observed a reduction in the expression of several genes involved in the immune "Signaling by Interleukin" REACTOME pathway early in the acute phase of pCHIKV-CIJD patients. Among these, *CCR2*, the gene encoding for an immune receptor is involved in inflammatory responses and the recruitment and activation of immune cells. Alterations in the CCR2 signaling pathway have been implicated in pathological conditions, including rheumatoid arthritis[17]. In this context, a 2014 study challenged CCR2 knockout mice with CHIKV infection. The authors observed that CCR2 deficiency impaired the immune response, reducing pro-inflammatory cytokine production. This imbalance in cytokine production may contribute to the chronic and dysregulated immune response observed in chikungunya virus-induced arthritis[18]. Here, although not

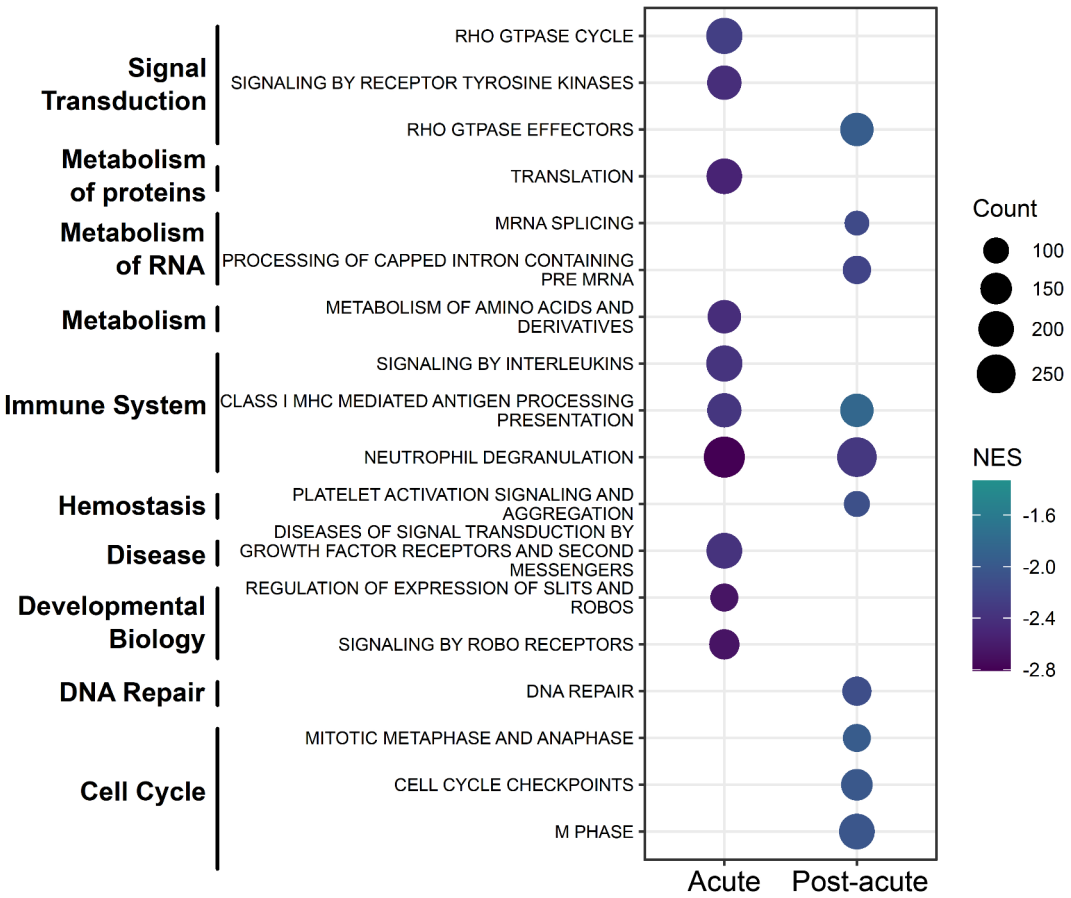

Fig. 2. Top 10 REACTOME pathways enriched in pCHIKV-CIJD patients. Functional enrichment analysis of expressed genes revealed the top 10 REACTOME pathways enriched in patients with Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD). Each dot represents a pathway, with the size indicating the number of enriched genes. The color gradient reflects the Negative Normalized Enrichment Score (NES) value, with warmer colors representing greater enrichment significance. Since all enriched pathways have negative NES values, warmer colors indicate pathways with stronger down-regulation in pCHIKV-CIJD patients.

significantly, it is possible to observe a reduction in *CCR2* expression (Log2FC = – 0.42; FDR = 0.19; Table S4) in patients who progressed to pCHIKV-CIJD, corroborating data found by others in vivo.

Another gene in the "Signaling by Interleukin" enrichment core, *LIFR* , shows a significant reduction in expression in pCHIKV-CIJD patients compared to non-pCHIKV-CIJD patients (Log2FC = – 2.12; FDR = 0.08, Table S1a). *LIFR* encodes the leukemia inhibitory factor (LIF) receptor, which, in complex with gp130, activates a tyrosine kinase signaling cascade in response to its ligand LIF. This pathway is key in coordinating immune responses with both pro- and anti-inflammatory functions[19]. This signal transduction pathway dysregulation is associated with many pathological conditions[20].

Interestingly, a study published in 2017 describes an autocrine regulatory network orchestrated by LIF/LIFR. Briefly, LIFR/gp130 forms a transmembrane complex with JAK1 and TYK2, which promotes the phosphorylation of the STAT4 transcription factor (pSTAT4) in the presence of the LIF. Then, pSTAT4 enhances the transcription of key inflammatory mediators, including IL-6, IL-8, IL-1α, IL-1β, and granulocyte-colony stimulating factor (G-CSF)[21]. In our study, non-pCHIKV-CIJD patients exhibited a higher *LIFR* transcription in the acute phase compared to pCHIKV-CIJD individuals. We hypothesize that this expression may result in higher LIFR availability, leading to increased LIF binding and signaling cascade activation. This, in turn, could enhance the transcription of inflammatory mediators, potentially contributing to antiviral immune responses in the early stages of the disease. This improved antiviral activity could help resolve the infection, reducing the risk of the disease becoming chronic in these patients. In contrast, the low amount of *LIFR* transcripts in the acute phase of infection, as observed in the pCHIKV-CIJD group, could result in the opposite effect, contributing to viral persistence that would increase the risk of chronicity.

During the acute phase, additional alterations were observed in pathways related to Signal Transduction, Metabolism, Immune System, and Development Biology. Notable differentially expressed genes (DEGs) within these pathways included Albumin (*ALB*), Leukemia Inhibitory Factor Receptor (*LIFR*), Necdin (*NDN*), and Zinc Finger and BTB Domain Containing 16 (*ZBTB16*). Furthermore, a widespread reduction in the transcriptional levels of genes associated with antiviral immune responses, interleukins and their receptors, and genes involved in immune cell recruitment and adaptive responses were evident in pCHIKV-CIJD patients. These findings are integral to the understanding of the dysregulated immune responses characteristic of chronic CHIKV infection.

In the post-acute phase, GSEA revealed enriched pathways associated with Signal Transduction, RNA Metabolism, Homeostasis, DNA Repair, and Cell Cycle. Noteworthy DEGs within these pathways included Anaplastic Lymphoma Receptor Tyrosine Kinase (*ALK*) and Protein C Cofactor (*PROS1*), which play crucial roles in DNA repair and Platelet Activation Signaling and Aggregation, respectively. These pathways shed light on the ongoing molecular processes contributing to the pathogenesis of pCHIKV-CIJD in the post-acute phase.

In both the acute and post-acute phases, two immune system pathways, Neutrophil Degranulation and Class I MHC Mediated Antigen Processing and Presentation, exhibited significant down-regulation in pCHIKV-CIJD patients.

Neutrophil degranulation involves the release of biological mediators stored in intracellular granules, including enzymes, cytokines, and antimicrobial molecules. Scientific studies have demonstrated the importance of this pathway in the response to viral infections. Opasawatchai et al. investigated the immune response during Dengue Virus infection and observed that neutrophil degranulation was critical for viral clearance and modulation of the inflammatory response[22]. Chikungunya patients also increased the neutrophil degranulation pathway in the acute phase of the disease[23,24]. Considering its crucial role in viral elimination, reducing genes that participate in degranulation activity can decrease viral clearance and raise the risk of viral dissemination, enabling wider systemic viral dissemination. Furthermore, an impaired degranulation process may decrease the release of pro-inflammatory cytokines, which can impact the activation of other immune cells and the coordination of the immune response.

Class I MHC-mediated antigen Processing and Presentation is critical to the host defense against viral infections, including CHIKV[25]. Here, pCHIKV-CIJD patients showed reduced levels of *ZBTB16* transcripts (Log2FC = – 1.15; FDR = 0.06; Table S1a), encoded for PLZF (promyelocytic leukemia zinc finger), in the acute phase. PLZF is a transcriptional factor that directly influences the expression of a specific set of genes induced by interferon cytokines. It can also influence the immune system's ability to control viral replication and contain the spread of the infection[26]. Furthermore, there is evidence that *ZBTB16* is necessary for the proper differentiation, survival and function of human T cells[27]. Therefore, a reduction in the ZBTB*16* transcription could impact the ability of T cells to eliminate virus-infected cells.

From the acute to the post-acute phases, pCHIKV-CIJD patients have shown a reduction in the expression levels of class I Human Leukocyte Antigen (HLA), such as *HLA-B, HLA-C, HLA-E, and HLA-F*, which are essential for effective immune responses against pathogens[28]. HLA, also known as MHC, proteins are expressed on the surface of cells, and their main function is to present virus peptide fragments to immune system cells like T CD8 + lymphocytes. This antigen presentation is essential for properly activating the immune system, allowing it to identify and eliminate infected cells. Interestingly, the suppression of *HLA* expression was recently described as an evasion mechanism in viral infections[29].

This set of results seems to indicate that, early in the acute phase, alteration of at least three key mechanisms in pCHIKV-CIJD patients may impair their immune response, possibly leading to an increase in viral persistence (Fig. 6), already described as a risk factor for chronification[30].

A detailed breakdown of all genes within the enrichment core of the top ten REACTOME pathways, alongside their corresponding statistical parameters, can be found in Table S3.

MicroRNA expression analysis reveals dysregulated regulatory networks in pCHIKV-CIJD patients

In this study, alongside total RNA sequencing, we conducted small RNA sequencing using the Illumina platform to assess the expression of microRNAs (miRNAs) in patients with Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD). Integrating miRNA expression data with total RNA sequencing offers valuable insights into post-transcriptional regulatory mechanisms and enhances our understanding of gene expression dysregulation in disease states.

The differential expression analysis of miRNAs revealed significant dysregulation in pCHIKV-CIJD patients compared to non-pCHIKV-CIJD individuals (Table S1b). In the acute phase, we identified 55 differentially expressed miRNAs (DEMs), include 21 up-regulated and 34 down-regulated miRNAs (Fig. 3A). Similarly, 73 DEMs were identified in the post-acute phase, comprising 35 up-regulated and 38 down-regulated miRNAs (Fig. 3B). These dysregulated miRNAs may play critical roles in modulating gene expression patterns associated with the pathogenesis of pCHIKV-CIJD. Additionally, a heatmap illustrating the hierarchical clustering of DEMs in both the acute (Fig. 3C) and post-acute phases (Fig. 3D) provides further insights into the dynamics of miRNA expression during different stages of the disease. Although not wholly segregating, the Hierarchical clustering heatmap revealed discernible expression profiles among patients with the same outcomes, suggesting potential associations between miRNA expression patterns and disease severity or progression. The complete list of identified mature miRNAs, along with their count information for each library, is provided in Table S5.

The dysregulation of miRNAs observed in pCHIKV-CIJD patients provides insights into potential regulatory networks involved in the disease process. We conducted an interactome analysis to elucidate these networks, considering both DEMs and differentially expressed genes (DEGs) in the acute and post-acute phases. All identified regulatory networks can be visualized in Fig. 4, providing a comprehensive overview of the interactions between dysregulated miRNAs and their target genes in pCHIKV-CIJD patients.

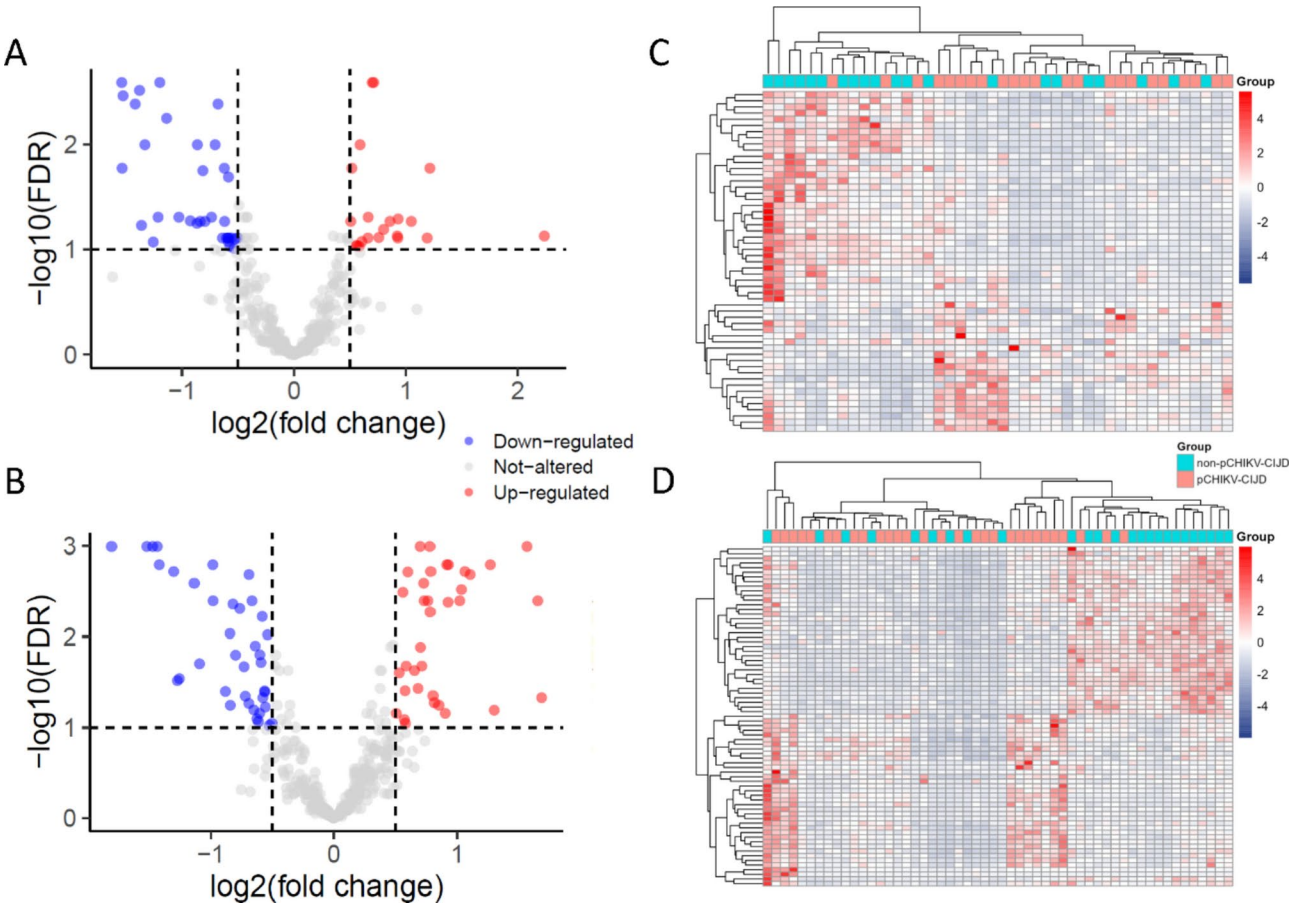

Fig. 3. Differential expression analysis of microRNAs (miRNAs) in pCHIKV-CIJD patients. (**A**) Volcano Plot representing the differentially expressed microRNAs (DEMs) found in patients with Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD) compared to non-pCHIKV-CIJD individuals in the acute phase of infection. (**B**) Volcano Plot representing the DEMs found in pCHIKV-CIJD patients compared to non-pCHIKV-CIJD individuals in the post-acute phase of infection. (**C**) A hierarchical clustering heatmap depicts the global expression profile of DEMs between pCHIKV-CIJD and non-pCHIKV-CIJD samples in the acute phase of infection. (**D**) A hierarchical clustering heatmap depicts the global expression profile of DEMs between pCHIKV-CIJD and non-pCHIKV-CIJD samples in the post-acute phase of infection.

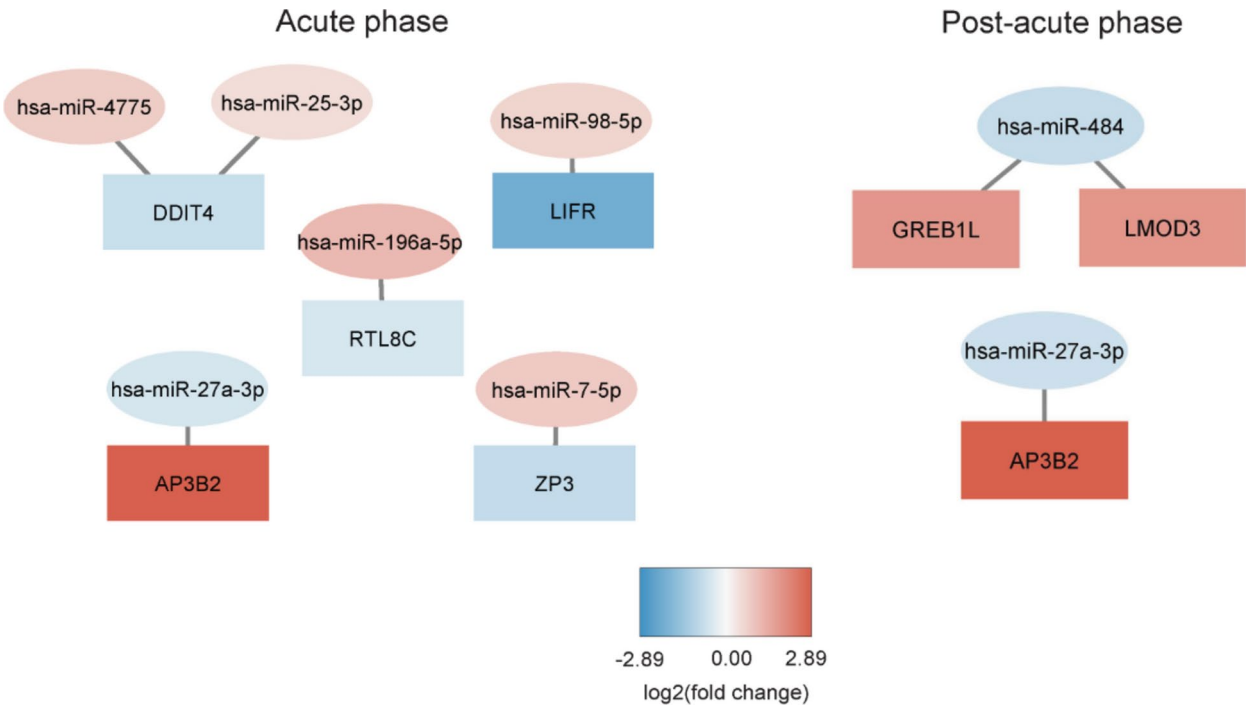

Fig. 4. Regulatory networks involving differentially expressed microRNAs (DEMs) and genes (DEGs) in acute and post-acute phases. This interactome illustrates the regulatory networks involving DEMs and DEGs that are differentially expressed in both acute and post-acute phases of post-Chikungunya chronic Inflammatory Joint Disease (pCHIKV-CIJD). MicroRNAs are represented by ellipses, while target genes are depicted as rectangles. The color gradient ranging from blue to red represents the Log2 Fold Change value, indicating the extent of differential expression. Lines connecting microRNAs and targets represent interactions between them. These regulatory networks provide insights into the complex interplay between microRNAs and their target genes, highlighting potential regulatory mechanisms involved in the pathogenesis of pCHIKV-CIJD across different phases of the disease.

Five potential regulatory networks between miRNAs and their validated target genes were identified in the acute phase of the disease.

One notable interaction identified was between the up-regulated miRNA hsa-miR-98-5p and the down-regulated target gene *LIFR*. The low levels of *LIFR* transcripts observed in the acute phase of pCHIKV-CIJD patients, as discussed earlier, can be partly explained by the increased expression of hsa-miR-98-5p in these patients, which is an experimentally validated regulator of *LIFR*[31]. Interestingly, hsa-miR-98-5p was already described as an estradiol (E2)-responsive miRNA. In vitro experiments using breast cancer cell culture showed that E2 induces 21 microRNAs, including hsa-miR98-5p[32]. E2 is described as the main female sex hormone and steroid which when binding to its receptors (ER), promotes the regulation of a series of biological processes, including the activation of gene expression[33,34]. Sex hormones may play a crucial role since they can potentially regulate many cellular processes, including the transcription of microRNAs, which in turn regulate the translation of thousands of targets[35]. Indeed, gender differences in response and prognosis are evident in several viral diseases. For instance, during the COVID-19 pandemic, men experienced higher mortality rates compared to women[36].

Studies have shown that women have at least a 50% increase in the risk of developing post-chikungunya chronic disease[5]. Of the 29 pCHIKV-CIJD patients from whom we obtained transcriptome data, 25 were women. Notably, non-pCHIKV-CIJD patients were selected for sequencing based on sex and age matching. Therefore, the differences in the expression of hormonally induced microRNAs and other related genes may be even greater in the general population.

Another interesting interaction between the up-regulated miRNAs hsa-miR-4775 and hsa-miR-25-3p with the down-regulated target genes *DDIT4*. Notably, hsa-miR-4775 has been experimentally validated to target the *DDIT4* gene, which exhibits a 68% reduction in expression earlier in chronic patients (Log2FC = – 0.75; FDR = 0.06; Table S1). *DDIT4* encodes the REDD1 protein, known for its regulatory role in cellular stress response and as a canonical mTOR regulator[37]. Furthermore, REDD1 has been implicated in the regulation of autophagy through a mTOR-independent mechanism, leading to the induction of reactive oxygen species and activation of the *ATG4B* gene, a crucial component of the autophagy machinery[38].

Previous studies have demonstrated high expression levels of REDD1 in cartilaginous tissue cells in both humans and rats. However, it has been observed that REDD1 expression significantly declines with age and in animal models of osteoarthritis, suggesting a potential involvement in the pathogenesis of this condition. The decreased expression of REDD1 in cartilage may contribute to enhanced mTOR activity and diminished

autophagy, which are characteristic features of osteoarthritis. Remarkably, lower expression levels of REDD1 have been associated with more severe inflammatory conditions in osteoarthritis[39].

Similarly, other up-regulated miRNAs, including hsa-miR-196a-5p and hsa-miR-7-5p, were found to interact with down-regulated target genes such as *RTL8C* and *ZP3*, respectively. In contrast, down-regulated miRNAs hsa-miR-27a-3p showed interactions with up-regulated target gene *AP3B2*, and this regulatory interaction was identified both in the acute and post-acute phases, highlighting the dynamic nature of gene regulatory processes during the course of pCHIKV-CIJD.

Two targets were found for the down-regulated hsa-miR-484. The gene Growth Regulating Estrogen Receptor Binding 1 Like (*GREB1L*) is involved in immune cell infiltration in lung adenocarcinoma, and its methylation level is related to immune response and cytolysis in gastric adenocarcinoma[40]. The other target *LMOD3* belongs, to the Leiomodin protein family, and is associated with Nemaline myopathy, characterized by muscle weakness, hypotonia, and the presence of nemaline bodies in skeletal muscle fibers[41].

The interactome analysis provides us critical insights into the regulatory networks influenced by miRNAs in pCHIKV-CIJD. By revealing complex interactions between dysregulated miRNAs and their target genes, this approach enhanced our understanding of the molecular mechanisms underlying disease progression and offers a novel avenue for future studies in the field.

Gene co-expression modules found in pCHIKV-CIJD patients

Gene co-expression analysis is a powerful approach for identifying correlated gene expression patterns across different samples or conditions. It enables the identification of functional gene modules that are co-regulated and play similar biological roles in cellular or pathological processes. In this study, we analyzed gene co-expression using total RNA sequencing data to identify gene co-expression modules associated with Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD). The results of this analysis provided insights into the molecular mechanisms underlying disease pathogenesis, as well as identify potential biomarkers or therapeutic targets.

The co-expression network analysis detected thirteen functional modules with similar gene expression, ranging from 42 to 1095 genes in each module. These modules were classified and named based on the number of genes they comprised (as shown in Figure S2). We observed that modules M1, M2, and M10 exhibited higher gene expression, while modules M4, M6, M7, and M11 showed lower gene expression in the acute phase of the disease compared to the post-acute phase.

Three modules (M3, M12, and M13) exhibited distinct behavior between pCHIKV-CIJD and non-pCHIKV-CIJD patient groups. Module M3 showed higher activity in pCHIKV-CIJD and lower activity in non-pCHIKV-CIJD patients, encompassing 886 genes, including 5 hubs (Fig. 5A). The top pathways of module M3 in the over-representation analysis involved in RNA metabolism, such as the processing of tRNAs and rRNAs in mitochondria (Fig. 5B). Conversely, modules M12 and M13 showed less expression in pCHIKV-CIJD and greater expression in non-pCHIKV-CIJD patients in both acute and post-acute phases of the disease. Module M12 included 46 hub genes (Fig. 5C) and was associated with the HDMs demethylate histones pathway (Fig. 5D). In comparison, module M13 comprised 42 genes, including 5 hubs (Fig. 5E), and was associated with Neutrophil Degranulation, Antimicrobial Peptides, Activation of Matrix Metalloproteinases, and Collagen Degradation pathways (Fig. 5F).

The set of genes co-expressed in the M13 module (Fig. 5E) had reduced activity in pCHIKV-CIJD patients in both disease phases. These genes are enriched in the activation of matrix metalloproteinases and collagen degradation pathways, already associated with the development and/or higher severity of other types of arthritis, such as rheumatoid arthritis and osteoarthritis[42]. One of the M13 hubs is *MMP8* (Fig. 5E), encoding matrix metalloproteinase 8, which was described as playing a protective role in the progression of inflammatory arthritis in mice. *MMP8* deficiency led to a reduction in caspase 11 expression, involved in programmed cell death, which can result in a delay in neutrophil apoptosis, subsequently causing neutrophil hyper infiltration in joints, contributing to the persistence of joint inflammation and an exacerbation in the severity of inflammatory arthritis[42].

Another M13 hub is *LFT*, which encodes lactoferrin, a protein present in many biological fluids capable of performing various biological functions such as immune response modulation, antimicrobial activity, and anti-inflammatory effect[43]. More than 20 years ago, a study highlighted the potential of local administration of lactoferrin in reducing articular inflammation in mice autoimmune and infectious arthritis models[44]. *LFT* was described as having an anti-apoptotic effect on IL-1β-induced chondrocytes and thus may be a promising novel therapeutic agent for osteoarthritis (OA). Chondrocyte apoptosis is mainly responsible for cartilage degeneration in OA[45]. Indeed, a recent study even observed that, in mouse models of rheumatoid arthritis, supplementation with bovine LFT considerably reduced inflammation and disease progression[46]. Considering the low expression of *LFT* found here since the early stages of the pCHIKV-CIJD, in addition to the large number of studies showing the broad physiological role played by lactoferrin, we can consider supplementation with lactoferrin as a possible treatment that can be applied even in acute phase of the disease aiming to mitigate the risks of disease progression, thus reducing the impacts on the quality of life of patients in the long term and, consequently, reducing expenses for the public health system. However, further studies are needed to evaluate this phenomenon in more patients.

These results provide valuable insights into the gene co-expression patterns associated with pCHIKV-CIJD, highlighting potentially relevant biological pathways and cellular processes for disease pathogenesis. Additionally, identifying distinct gene modules between patients with and without pCHIKV-CIJD suggests the existence of specific molecular signatures associated with disease progression and severity.

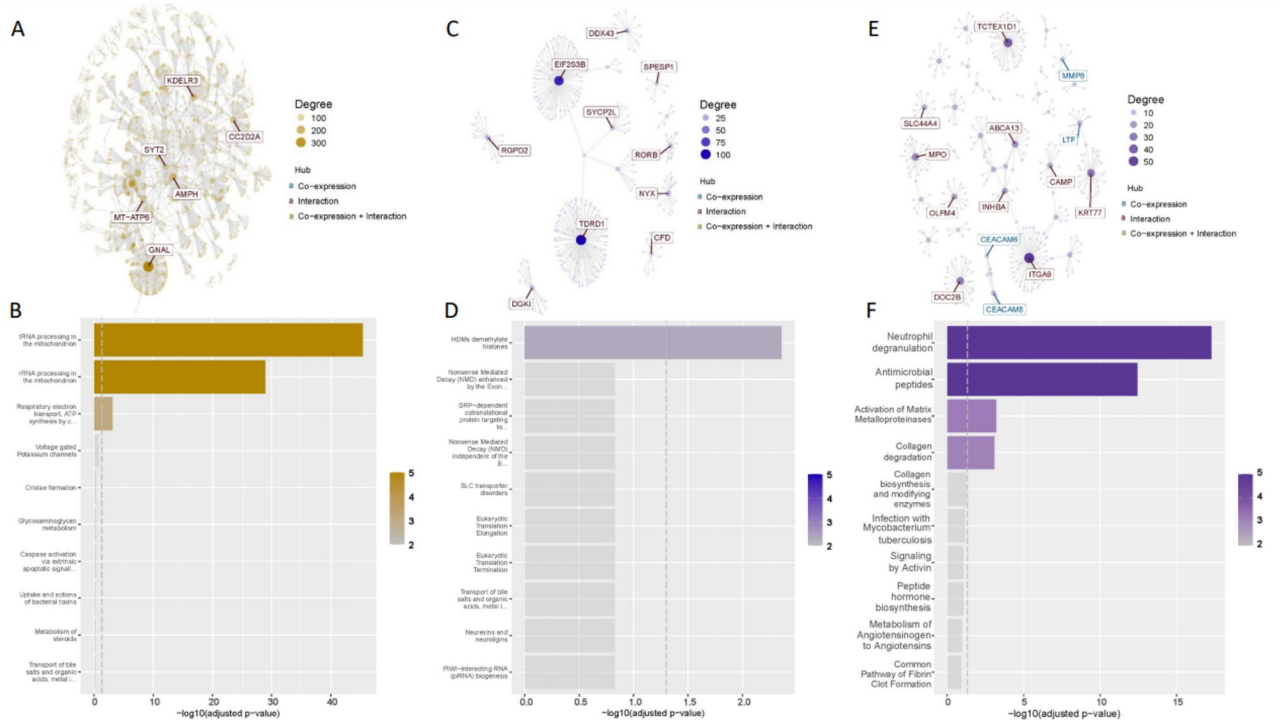

Fig. 5. Co-expression modules found in pCHIKV-CIJD patients. The three co-expression modules exhibited distinct patterns in patients with Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD). Gene networks of modules displaying the most connected genes (hubs) of M3 (**A**), M12 (**C**), and M13 (**E**) modules are illustrated. Each dot represents a gene, with the size and color reflecting the degree of interaction within the module. Larger dots and warmer colors indicate genes with higher connectivity within the module. Additionally, over-representation analysis was performed to identify pathways impacted by the genes within each module. The pathways affected by the genes of M3 (**B**), M12 (**D**), and M13 (**F**) modules are presented. In both analyses, the yellow color indicates a trend of greater expression in the genes of this module, while the purple color indicates a trend of lower expression in the genes of this module.

Conclusion

Responses to CHIKV infections involve complex mechanisms with intricate regulation of processes. To better understand these mechanisms, we aimed to investigate the factors involved in the progression of patients to post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD), a condition that affects a considerable proportion (25–50%) of symptomatic CHIKV fever patients. To achieve this, we performed total and small RNA sequencing from whole blood samples collected during the acute and post-acute phases of the disease in patients who either progressed or did not progress to pCHIKV-CIJD. These patients were part of a longitudinal cohort study that we recently published[5].

Overall, our findings provide critical insights into the molecular mechanisms underlying the progression to pCHIKV-CIJD. The identification of specific DEGs, particularly the high prevalence of lncRNAs, opens new avenues for understanding the pathogenesis of this chronic condition. These results highlight potential molecular targets for future research and therapeutic intervention to mitigate the chronic inflammatory processes associated with pCHIKV-CIJD.

In summary, here we show that patients who evolve to the chronic phase of the disease have a reduction in interleukin signaling in the initial phases of the disease compared to those who do not evolve. The impairment observed in this pathway seems to be explained at least in part by the increased expression of hsa-miR-98-5p, a miRNA activated by estradiol. It is important to note that estradiol is the main hormone produced by women, the population most affected by pCHIKV-CIJD. Furthermore, other antiviral immune response mechanisms in acute and post-acute phases seem to be impaired in these patients, such as neutrophil degranulation and antigen presentation via the MHCI pathways. Together, these mechanisms can lead to a reduction in the first line of antiviral response, promoting the persistence of the virus for a longer period in these patients (Fig. 6). This lack of immune response and other mechanisms such as reduced levels of matrix metalloproteinases (MMP8) and DDIT4, which appear to contribute to developing different types of arthritis, are important mechanisms for developing the post-Chikv chronic joint disease.

The study has limitations, including a small sample size, which may affect the generalizability of the findings, and the lack of data validation in an independent cohort due to the unavailability of samples.

Despite these limitations, we set out to generate and analyze small and total RNA data in two phases of infection in nearly 60 CHIKV-infected patients who were followed for 90 days and objectively diagnosed as to the outcome. This study provides valuable insights that can help to find a way to mitigate the progression of the

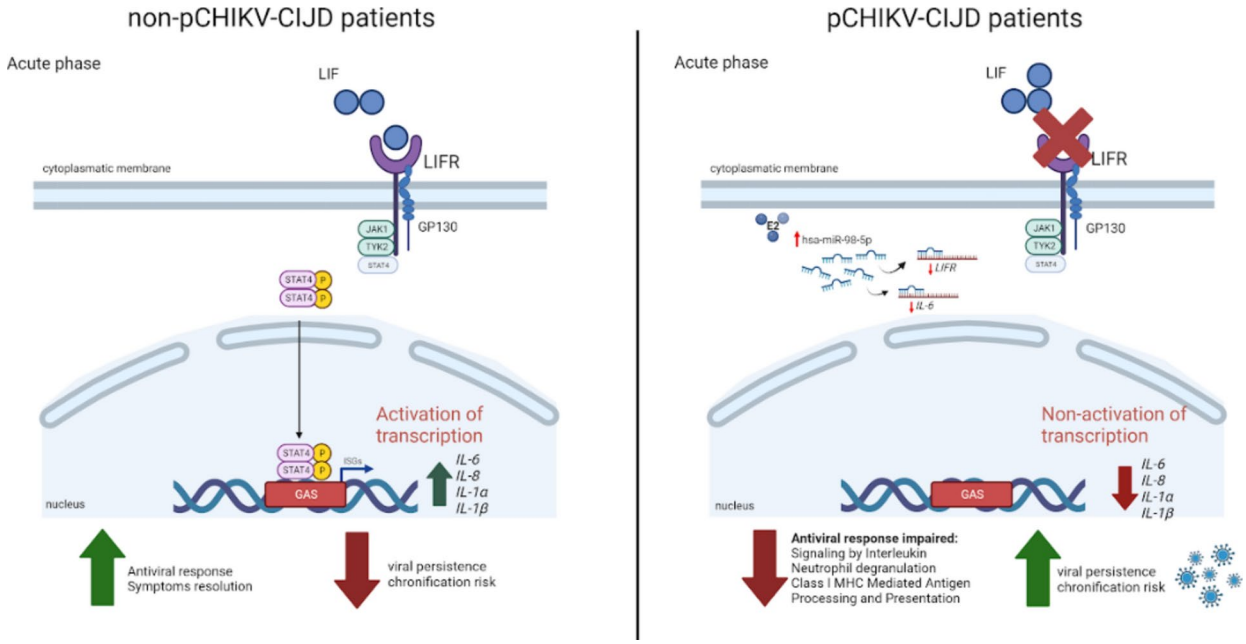

Fig. 6. Hypothetical Mechanism of *LIFR* Expression and Viral Persistence. Proposed diagram of the molecular alterations found still in the early stages of CHIKV infection in patients who progressed to post-chikungunya chronic inflammatory joint disease (pCHIKV-CIJD). Higher expression of LIF receptor (LIFR) may enhance LIF binding and activate downstream signaling pathways, leading to increased transcription of inflammatory mediators. This boost in antiviral immune responses could aid in resolving the infection and reducing the risk of chronic disease. Conversely, reduced LIFR transcript levels in the acute phase of Post-Chikungunya Chronic Inflammatory Joint Disease (pCHIKV-CIJD) could impair antiviral responses, contributing to viral persistence and a higher likelihood of chronicity. *E2* estradiol hormone. Created in BioRender. Sabino, E. (2025) https://BioRender.com/w73d110

disease to a more severe prognosis. In Brazil, a tropical country where the *Aedes aegypti* mosquito is endemic, chikungunya poses a significant public health risk, especially without an effective vaccine. The present study helps to elucidate the mechanisms involved in the evolution of patients to pCHIKV-CIJD. This disease significantly reduces the quality of life of affected individuals and financially impacts public health systems. Further studies must confirm these findings in a larger sample size and with different methodological approaches.

Methods

Sample selection

A prospective longitudinal cohort at the Evandro Chagas National Institute of Infectology—INI/FIOCRUZ—Rio de Janeiro-RJ was established in 2019, including patients who suspected CHIKV. The inclusion and exclusion criteria and clinical and epidemiological aspects of the cohort were described in a previous study published recently by Lazari and coworkers[5]. Briefly, patients were included with up to seven days of fever and classic arbovirus symptoms. Two follow-up visits were made, 21 (D21) and 90 days (D90) after inclusion (D0), where collections of biological material and clinical information about the pain of joint symptoms were carried out. Those patients who, at D90, remained with joint symptoms and signs of arthritis were referred for ultrasound imaging examination. The 29 cases that showed image alteration were classified as post-CHIKV Chronic Inflammatory Joint Disease (pCHIKV-CIJD). From 47 patients who completed the follow-up scheme and did not present pCHIKV-CIJD, 25 were selected as a comparison group. This pairing was made considering, as criteria, that there were no significant differences regarding the age (pCHIKV-CIJD age median = 49.5/non-pCHIKV-CIJD age median = 43; $p = 0.31$) and sex (pCHIKV-CIJD = 25 female and 4 male/non-pCHIKV-CIJD = 22 female and 11 male; $p = 0.08$) between the groups. Finally, samples from these patients were sequenced in the acute and post-acute phases, according to the availability and quality of clinical material. The number of samples from each disease phase by group that were sequenced is shown in Fig. 7 and the clinical information of the patients whose samples were sequenced is provided in Table S6.

Total and small RNA sequencing

For total RNA sequencing, the TruSeq Stranded Total RNA Library Prep kit with RiboZero Gold (Illumina, San Diego, California, USA) was used to prepare the libraries with an input of 500 ng. The RNA samples had a RIN (RNA Integrity Number) greater than 7 and/or a DV_{200} value greater than 70%. The libraries were sequenced using the NextSeq 500/550 High Output Kit v2.5 (150 Cycles) in a paired-end mode, generating reads of 2×75 bp. For small RNA sequencing, the TruSeq Small RNA Library Preparation Kit (including the miR-456 depletion step[47]) was used, and sequencing was performed with the NextSeq 500/550 High Output Kit v2.5 (75

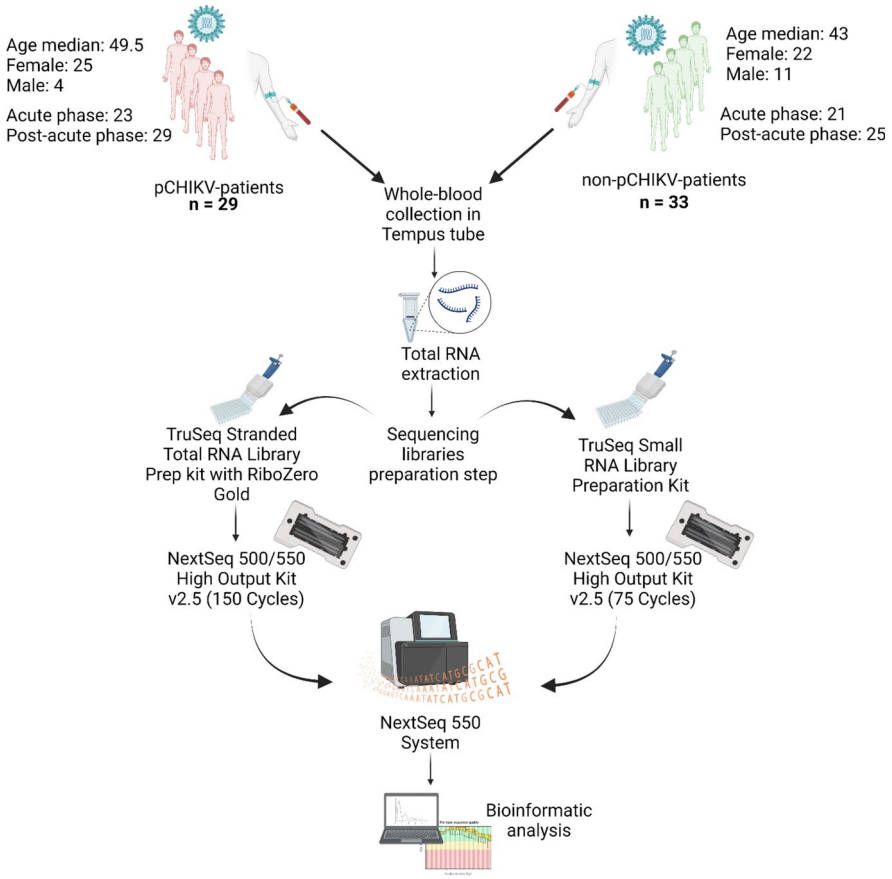

Fig. 7. Representative flowchart of the methods used in this study. *pCHIKV-CIJD* post-Chikungunya Chronic Inflammatory Joint Disease, *non-pCHIKV-CIJD* non-post-Chikungunya Chronic Inflammatory Joint Disease, *n* number of samples. Acute phase: Samples collected at inclusion (D0); post-acute phase: Samples collected until D21. Created in BioRender. Sabino, E. (2025) https://BioRender.com/t78w537

Cycles), generating single-end reads of 75 bp. A total of 98 libraries were made and 12 pooled samples were run simultaneously in each round on an Illumina NextSeq 550 (Illumina).

The Fastq files from the total and small RNA sequencing are submitted on the Gene Expression Omnibus (GEO) platform under the accession number PRJNA1001790 and the metadata of the individuals and the sample characteristics can be found in Table S6.

A summary of the study workflow is shown in Fig. 7.

Total RNA sequencing analysis

After sequencing, all the Fastq files were analyzed for the quality control of the reads using the FASTQC software[48]. The sequences were mapped to the human genome (GRCh38) using STAR software (v2.7)[49]. Gene count matrices were generated with the featureCounts function from the Rsubread package (v2.4.3)[50] in the R programming language (v4.0.5). Differentially expressed genes were identified using the edgeR package (v3.32.1)[51], through a comparison of pCHIKV-CIJD vs. non-pCHIKV-CIJD. A pathway enrichment analysis was performed using p-value and gene expression signal to rank the gene list and compare it with the Reactome pathways (v76)[52] information to identify pathways enriched in the expressed gene pool. This analysis was performed using the fGSEA package (v1.16.0)[53]. Finally, to identify genes with similar expression profiles between the sample classes (chronic early, chronic late, non-chronic early, non-chronic late), a coexpression analysis was performed using the CEMITool package (v1.14.1)[54].

Small-RNA sequencing analysis

The read quality control was accessed as described above. The sequences were mapped to the mature miRNA sequences from miRBase release 22[55] using bowtie2[56], which was chosen for its compatibility with updated software tools and efficient handling of small RNA reads. Gene count matrices were generated with the featureCounts[57] in the R programming language (v4.0.5). Differentially expressed microRNAs were identified using the edgeR package (v3.32.1)[51]. To evaluate the microRNA target genes, the miRTarBase database release 8.0[58] was used, which considers only those targets that have been experimentally validated.

Interactome

Only the validated target mRNAs with inverted log2FC patterns from their miRNAs were considered to build the miRNA-target networks. We used the interaction information of lncRNAs and protein-coding genes from the NPInter v5.0 database[59] to build a network for the genes found in this study. Only interactions with at least log2FC > |0.5| and adjusted p-value < 0.05 for one of the nodes were considered to create the network. All the networks in this study were created using the Cytoscape software[60].

The pipeline and associated scripts used for this study have been uploaded to GitHub and are accessible at https://github.com/ftencaten/pchikv_cijd_transcriptomics.

Data availability

Sequence data that support the findings of this study have been deposited in the NCBI SRA database with the primary accession code PRJNA1001790.

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Author contributions

MSR: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing—Original Draft Preparation, Writing—Review & Editing; GCF: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing—Original Draft Preparation, Writing—Review & Editing; FTC: Formal Analysis, Investigation, Methodology, Writing—Review & Editing; ALG: Methodology, Validation, Visualization, Writing—Review & Editing; APG: Methodology, Validation, Visualization, Writing—Review & Editing; ERM: Methodology, Validation, Visualization, Writing—Review & Editing; MFC: Methodology, Validation, Visualization, Writing—Review & Editing; GMP: Methodology, Validation, Visualization, Writing—Review & Editing; OB: Methodology, Validation, Visualization, Writing—Review & Editing; MGC: Methodology, Validation, Visualization, Writing—Review & Editing; CSL: Conceptualization, Data Curation, Visualization, Writing—Review & Editing; PB: Conceptualization, Data Curation, Investigation, Methodology, Supervision, Writing—Review & Editing; CSB: Conceptualization, Data Curation, Investigation, Methodology, Supervision, Writing—Review & Editing; HN: Conceptualization, Data Curation, Formal Analysis, Methodology, Validation, Visualization, Writing—Review & Editing; GPB: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Software, Resources, Validation, Supervision, Writing—Review & Editing; ATRV: Data Curation, Formal Analysis, Investigation, Methodology, Validation, Supervision, Writing—Review & Editing; PB: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Supervision, Writing—Review & Editing; ECS: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Software, Resources, Validation, Supervision, Writing—Original Draft Preparation, Writing—Review & Editing.

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Competing interests
The authors declare no competing interests.

Ethical approval
All methods were carried out in accordance with relevant guidelines and regulations. The experimental protocols were approved by the Ethical Review Board of the Faculdade de Medicina da Universidade de São Paulo (CAAE: 71611417.9.1001.0065; approval number: 2.262.437). Written informed consent was obtained from all participants and/or their legal guardians prior to their inclusion in the study.

Additional information
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