Original Research *Highlight article*

Identification in synovial fluid of a new potential pathogenic player in arthropathies

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Impact Statement

The stimulator of interferon genes (STING) is a transmembrane protein of the endoplasmic reticulum that behaves as a sensor of cytosolic DNA and leads to the production of IFN-I and pro-inflammatory cytokines. Although it is well known that STING plays an important role in innate immune responses, its potential involvement in rheumatic disease processes remains to be elucidated. This study demonstrates that STING was contained at high concentrations within cells in inflammatory synovial fluids, and extracellularly in non-inflammatory synovial fluids or in those with calcium pyrophosphate crystals. STING has never been identified in synovial fluids from psoriatic arthritis and intracellularly in osteoarthritis. In these same samples, STING mRNA levels were extremely low. Our results suggest that STING may play a key role in the pathogenesis and progression of arthropathies.

Abstract

STING (stimulator of interferon genes) has been recognized as an important signaling molecule in the innate immune response to cytosolic nucleic acids. Although it has been proposed that STING signaling pathway may play a pathogenic role in developing autoimmune and autoinflammatory diseases, its involvement in rheumatic disease processes remains to be elucidated. Here, we evaluated STING protein levels, expression and relationship with inflammatory parameters in synovial fluid (SF) of patients with psoriatic arthritis (PsA), rheumatoid arthritis (RA), gout, calcium pyrophosphate crystal-induced arthritis (CPP-IA), osteoarthritis (OA), and OA with CPP crystals (OA + CPP). The correlation with its negative regulator, nuclear factor erythroid 2-related factor 2 (Nrf2), was also investigated. SFs from 72 patients were analyzed for white blood cell (WBC) count, polymorphonuclear cell percentage (PMN%), and IL-1β, IL-6, IL-8, extra- and intracellular STING levels. STING and Nrf2 expression was also determined. WBC count and PMN% were greater in SF from inflammatory arthritis, while they were lower in OA groups. RA and gouty SFs have the highest levels of IL-1β, IL-8, and IL-6; while OA and OA + CPP showed the lowest concentrations. Gout and RA had the highest intracellular STING levels, while extracellular STING was greater in CPP-IA and OA SFs. STING was not detectable in PsA. STING mRNA was lower in PsA than other arthritides. Nrf2 mRNA was not detectable in OA. This study determines the presence of STING in SF of different arthritides, except for PsA, and suggests that it may be involved in pathogenesis and progression of arthropathies.

Keywords: Arthritis, cytokines, inflammation, STING, synovial fluid

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Introduction

The stimulator of interferon genes (STING) is a 40-kDa evolutionary conserved transmembrane protein of the endoplasmic reticulum (ER) which is ubiquitously expressed in several cells and tissues where it mainly facilitates the innate immunity signaling.[1] STING expression is downregulated by nuclear factor erythroid 2-related factor 2 (Nrf2) which operates as transcription factor involved in the antioxidative stress response and, when overexpressed, inhibits type I IFN response.[2]

STING behaves as a sensor of cytosolic DNA from bacteria and viruses and stimulates the production of type I interferon (IFN-α and IFN-β).[3] It has been demonstrated that STING recognizes and binds to cyclic dinucleotides: cyclic di-GMP (c-di-GMP), a second messenger released by bacteria, and cyclic GMP-AMP (cGAMP), a signaling molecule produced by cyclic GMP–AMP synthase (cGAS) in response to the presence of virus DNA in the cytosol.[4,5] Upon binding, STING oligomerizes, translocates from the ER to perinuclear compartments and is phosphorylated by the kinase TBK1, leading to enrollment and activation of the transcription factor IRF3 to promote expression of type I interferon and pro-inflammatory cytokines, such as TNF-α and IL-6.[6–8]

Beside a mediated IFN I immune response, STING plays a direct role in autophagy and promotes cellular senescence and apoptosis after DNA damages or microbial infection.[6]

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Different studies have reported the presence of IFN I in the synovial fluid (SF) of patients with rheumatic diseases,[9–11] thus suggesting a possible involvement of STING in the pathogenetic processes of these diseases. In particular, significant levels of INF-α have been detected in SF obtained from patients with rheumatoid arthritis (RA),[10] while INF-β was difficult to determine due to its high instability in this body fluid.[9] More recently, it has been found that STING may be involved in the development of autoimmune and autoinflammatory rheumatic diseases, such as systemic lupus erythematosus (SLE), and STING-associated vasculitis with onset in infancy (SAVI), and could be a central mediator of the downstream events leading to disease symptoms and inflammation.[12–14] These findings have been corroborated in murine models showing how targeting STING proteins may reduce overexpression of pro-inflammatory cytokines and severe spontaneous autologous DNA-mediated polyarthritis.[15] More recently, Hwang *et al.*[16] observed an activation of the cGAS-STING pathway triggered by degradation fragments of the extracellular matrix of cartilage (e.g. fibronectin fragments) and an overexpression of pro-inflammatory cytokines in SF samples of osteoarthritis (OA) patients. No studies to date have investigated the role of the STING pathway in other rheumatic inflammatory diseases. Therefore, we endeavored to evaluate the concentrations and expression of STING, as well as its correlation with local inflammation in SF of patients with psoriatic arthritis (PsA), RA, gout, calcium pyrophosphate crystal-induced arthritis (CPP-IA), OA, and OA with CPP crystals (OA + CPP). The downregulation of STING by Nrf2 has also been investigated as a potential hallmark in the pathogenesis and progression of joint diseases.

Materials and methods

Collection and analysis of synovial fluids

Human SF samples from patients were collected with the approval of the Institutional Review Board of the Padova University Hospital. All participants gave written informed consent. SF was collected by arthrocentesis from the knees of 72 untreated patients: 12 with PsA, 12 with RA, 12 with gout, 12 with CPP-IA, 12 with OA, and 12 with OA + CPP. SF samples were examined under optical light microscopy to determine total white cell count (WBC) and differential cell count. Pathogenic crystals (monosodium urate (MSU) and CPP crystals) were identified using ordinary and polarized light microscopy.[17] Patients' characteristics are outlined in Supplementary Table 1. SF cells and supernatants were stored at -80°C after centrifugation at 300*g* for 30 min as previously described.[18]

Determination of STING and cytokine concentrations

SF supernatants were analyzed to determine extracellular concentrations of IL-8, IL-6, IL-1β (eBioscience, San Diego, CA, USA) and STING (MyBioSource, Inc., San Diego, CA, USA) levels by enzyme-linked immunosorbent assay (ELISA). Intracellular STING concentrations were determined in lysates obtained after three freeze–thaw cycles and resuspended in phosphate-buffered saline (PBS).[19]

mRNA expression analysis

Total RNA was isolated with Total RNA Purification Kit (Norgen Biotek Corp., Canada) from human SF cells (six SFs per group). A pair of human STING primers, sense 5′-CCTGAGTCTCAGAACAACTGCC-3′ and anti-sense 5′-GGTCTTCAAGCTGCCCACAGTA-3′, of human Nrf2 primers, sense 5′-CTTTTGGCGCAGACATTCC-3′ and anti-sense 5′-AAGACTGGGCTCTCGATGTG-3′, and of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers, sense 5′-AGCCACATCGCTCAGACA-3′ and anti-sense 5′-GCCCAATACGACCAAATCC-3′, as housekeeping gene, were used for real-time reverse transcription polymerase chain reaction (RT-PCR) (ABI Prism 7900 HT, Thermo Fisher Scientific, Madison, WI, USA). iScript cDNA Synthesis Kit (Biorad, CA, USA) was used to convert 116 ng of total RNA into first-strand cDNA. Subsequently, RT-PCR reaction was run at the following amplification cycle: 95°C for 30 s, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min using iTaq Universal SYBR Green Supermix (BioRad, CA, USA). All molecular targets were analyzed in duplicates for each sample. Results were normalized to the housekeeping gene and evaluated using the 2-ΔΔCt method.

Statistical analysis

All values are expressed as mean ± SD. Data significance were evaluated by a one-way analysis of variance (ANOVA) followed by Dunnett's test, where appropriate. Correlations between different variables were calculated by Spearman rank correlation test. A p-value < 0.05 is considered significant.

Results

SF characteristics and analysis

The results of SF analysis are reported in Figure 1. SF from patients with inflammatory arthritis showed WBC count greater than 5000 cells/mm^3 with greater than 40% polymorphonuclear (PMN) cells. The highest concentrations of both parameters were observed in SFs from gout patients (WBC $26.74 \pm 18.43 \times 10^3$/mm^3; PMN 86% ± 9.86%). By contrast, WBC count and PMN% were lower in OA and OA + CPP patients (<500 cells/mm^3; PMN < 4%).

SF concentrations of cytokines

Similar cytokine profiles were observed in SF from patients with RA and gout, who showed the highest levels of IL-1β, IL-8, and IL-6, whereas patients with OA and OA + CPP showed the lowest concentrations of the same cytokines (Figure 2). SF concentration of IL-1β in gout and RA was eightfold to ninefold higher than in PsA, and sevenfold to eightfold higher than in CPP-IA. SF concentrations of IL-8 were threefold to fourfold higher in patients with RA and gout versus PsA and CPP-IA. SF concentrations of IL-8 in patients with OA + CPP were twofold that of patients with OA. Finally, SF concentration of IL-6 was fourfold to fivefold higher in patients with RA and gout versus PsA and CPP-IA.

SF concentrations of STING

Patients with gout and RA had the highest concentrations of intracellular STING (Figure 3(a)). STING was not

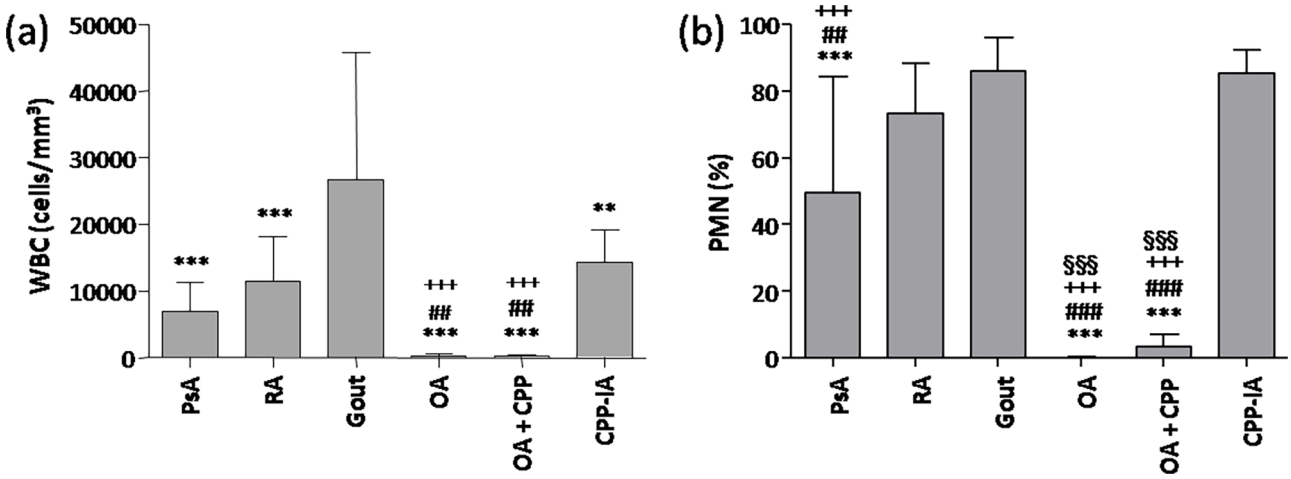

Figure 1. Cell counts in synovial fluids. (a) Number of white blood cells (WBC) and (b) percentage of polymorphonuclear cells (PMNs) were determined in synovial fluid (SF) from patients with psoriatic arthritis (PsA), rheumatoid arthritis (RA), gout, calcium pyrophosphate crystal-induced arthritis (CPP-IA), osteoarthritis (OA), and OA with CPP crystals (OA + CPP). Values are expressed as mean ± SD of 12 SF per group. $^{**}p < 0.01$ versus gout, $^{***}p < 0.001$ versus gout, $^{\#\#}p < 0.01$ versus RA, $^{\#\#\#}p < 0.001$ versus RA, $^{+++}p < 0.001$ versus CPP-IA, $^{\S\S\S}p < 0.001$ versus PsA.

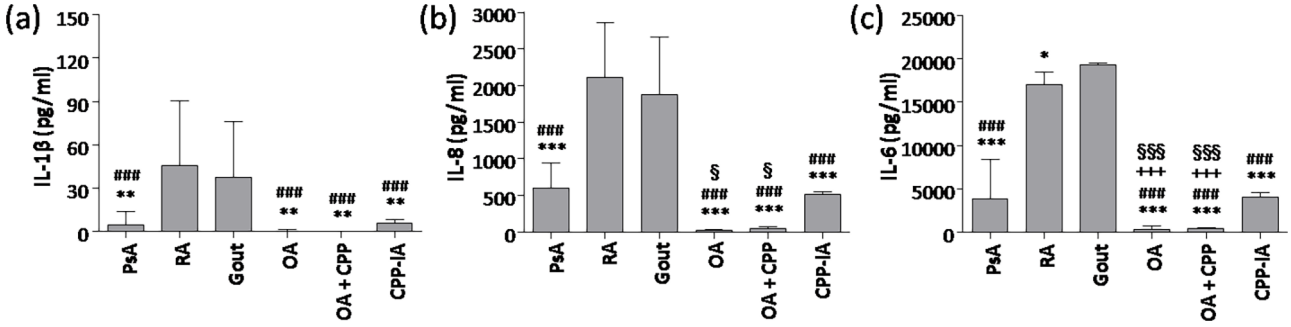

Figure 2. Cytokine concentrations in synovial fluids. (a) IL-1β, (b) IL-8, and (c) IL-6 concentrations were determined in synovial fluid (SF) from patients with psoriatic arthritis (PsA), rheumatoid arthritis (RA), gout, calcium pyrophosphate crystal-induced arthritis (CPP-IA), osteoarthritis (OA), and OA with CPP crystals (OA + CPP). Values are expressed as mean ± SD of 12 SF per group. $^{*}p < 0.05$ versus gout, $^{**}p < 0.01$ versus gout, $^{***}p < 0.001$ versus gout, $^{\#\#\#}p < 0.001$ versus RA, $^{+++}p < 0.001$ versus CPP-IA, $^{\S}p < 0.05$ versus PsA, $^{\S\S\S}p < 0.001$ versus PsA.

detectable in SF of PsA, OA, and OA + CPP patients. The highest concentrations of extracellular STING were found in SF of patients with CPP-IA and OA, which were twofold higher than in OA + CPP (Figure 3(b)). Extracellular STING remained under detection limit (50 pg/mL) in SF from RA, PsA, and gout.

We found a positive correlation between intracellular STING and WBC ($r = 0.631$; $p < 0.001$), PMN ($r = 0.703$; $p < 0.001$), IL-1β ($r = 0.784$; $p < 0.001$), IL-8 ($r = 0.657$; $p < 0.001$) and IL-6 ($r = 0.717$; $p < 0.001$) in SF. By contrast, we found a negative correlation between extracellular STING and IL-1β ($r = -0.454$; $p < 0.001$), IL-8 ($r = -0.640$; $p < 0.001$), and IL-6 ($r = 0.506$; $p < 0.001$) in SF.

SF STING and Nrf2 expression

As shown in Figure 4(a), the mRNA levels of STING were twofold, fivefold, and sixfold lower in PsA than in RA, gout, and CPP-IA, respectively; though it was similar in OA groups. Nrf2 mRNA was 2.5-fold higher in cells from RA, gout, and CPP-IA SFs compared with PsA (Figure 4(b)). Nrf2 was not detectable in SF from patients with OA without CPP crystals.

Discussion

Our results showed that STING was contained at high concentrations within cells in inflammatory SF, and extracellularly in non-inflammatory SF or in those with CPP crystals. STING has never been identified in SF from PsA and intracellularly in OA, which may explain the low expression of STING mRNA in SF of these patients, displaying a 2.5-fold downregulation versus RA, gout, and CPP-IA.

There is growing evidence that STING may play a relevant role in modulating inflammatory response and other pathophysiological processes involved in several diseases.[13] Elevated concentrations of STING were found in liver tissues of subjects with non-alcoholic fatty liver disease (NAFLD).[20] The increased expression of STING in peripheral blood mononuclear cells and intestinal biopsies of patients with abdominal sepsis, correlated with intestinal inflammation.[21] Elevated concentrations of STING were also found in endometrial epithelial cells of patients with endometriosis and adenomyosis, and correlated with inflammatory cell infiltration within the epithelium, thus indicating that STING signaling may play a role in initiating chronic inflammation.[22] Elevated STING concentrations were also found in

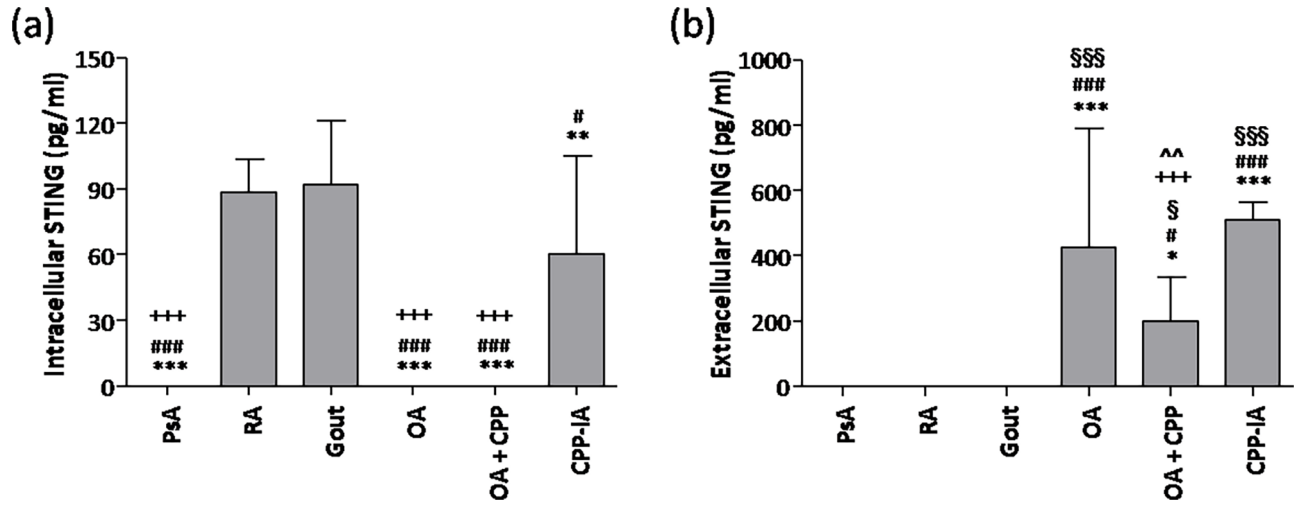

Figure 3. STING concentrations in synovial fluids. (a) intracellular and (b) extracellular STING concentrations were determined in synovial fluid (SF) from patients with psoriatic arthritis (PsA), rheumatoid arthritis (RA), gout, calcium pyrophosphate crystal-induced arthritis (CPP-IA), osteoarthritis (OA), and OA with CPP crystals (OA + CPP). Values are expressed as mean ± SD of 12 SF per group. $^{*}p < 0.05$ versus gout, $^{**}p < 0.01$ versus gout, $^{***}p < 0.001$ versus gout, $^{\#}p < 0.05$ versus RA, $^{\#\#\#}p < 0.001$ versus RA, $^{+++}p < 0.001$ versus CPP-IA, $^{\S}p < 0.05$ versus PsA, $^{\S\S\S}p < 0.001$ versus PsA, $^{\wedge\wedge}p < 0.01$ versus OA.

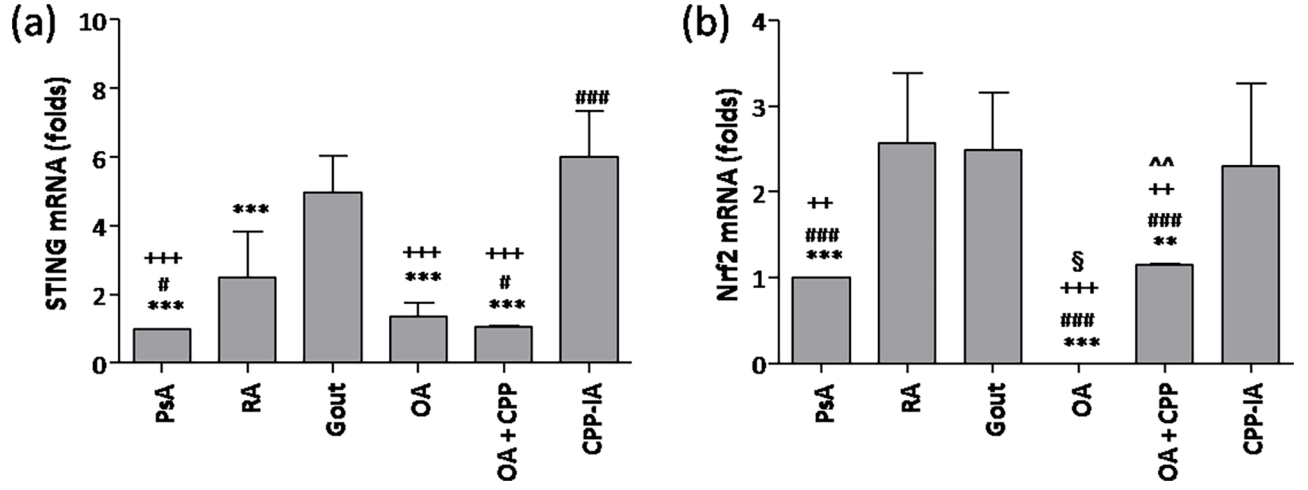

Figure 4. STING and Nrf2 expression in synovial fluids. (a) STING and (b) Nrf2 mRNA levels were determined in synovial fluid (SF) cells from patients with psoriatic arthritis (PsA), rheumatoid arthritis (RA), gout, calcium pyrophosphate crystal-induced arthritis (CPP-IA), osteoarthritis (OA), and OA with CPP crystals (OA + CPP). Values are expressed as mean ± SD of 12 SF per group. $^{**}p < 0.01$ versus gout, $^{***}p < 0.001$ versus gout, $^{\#}p < 0.05$ versus RA, $^{\#\#\#}p < 0.001$ versus RA, $^{++}p < 0.01$ versus CPP-IA, $^{+++}p < 0.001$ versus CPP-IA, $^{\S}p < 0.05$ versus PsA, $^{\wedge\wedge}p < 0.01$ versus OA.

colonic tissue of colitic mice as well as in murine or human M1-polarized THP-1 macrophages;[23] similarly, increased concentrations of STING mRNA and protein were found in leukocytes during acute pancreatitis in mice.[24] In line with the aforementioned findings, we demonstrated that elevated concentrations of intracellular STING in inflammatory SFs of patients with RA, gout, and CPP-IA patients, correlated with SF pro-inflammatory cytokine concentrations and with the cell infiltration. Conversely, intracellular STING remained under detection limits in SF from OA, which is associated with a lower degree of inflammation than other arthropathies considered.[25] STING plays a critical role in the release of Type I IFN in response to foreign pathogenic DNA and RNA, or mislocated self-DNA and mitochondrial DNA (mtDNA) in the cytosol.[15,26] Recent reports have proposed intracellular cell-free DNA (cfDNA) species (mitochondrial or nuclear) as potential biomarkers for autoimmune rheumatic diseases, such as RA. This stems from the observation that elevated concentrations of cfDNA have been found in body fluids of patients affected by these conditions and appear to be associated with cell death and inflammatory mechanisms, including apoptosis, necrosis, NETosis, and pyroptosis.[27,28] Increased concentrations of cfDNA in SF and serum of patients with RA, gout, and pseudogout may help explain our results.[29–31] Indeed, it has been demonstrated that cytosolic double-stranded DNA accumulates in fibroblast-like synoviocytes in patients with active RA and promotes synovial inflammation via the cGAS/STING pathway.[32] It is entirely possible that phagocytosed pathogenic crystals might interact directly with STING.

However, in our study, STING remained under detection limits in SFs of patients with PsA, despite inflammatory features. This indicates that STING might not be involved in the pathogenetic processes of PsA or a downregulation of

STING signaling. It has been reported that STING is tightly regulated by various control mechanisms, such as degradation, post-translational modifications, or overexpression of certain microRNAs that can target suppressor genes.[13,33] In PsA patients, in addition to specific serological biomarkers, suppressor genes have also been suggested as possible diagnostic and prognostic biomarkers as there have been reports of altered miRNA in articular tissues and peripheral blood mononuclear cells.[34,35]

In our study, we found extracellular STING in SF of patients with OA or CPP-IA. This may be due to the activation of processes that reduce its interaction with the ER and appears to corroborate previous reports showing that STING can be released from infected cells in microvesicles (e.g. exosomes).[36] An imbalance in cytosolic calcium concentrations, as seen in the presence of CPP or basic calcium phosphate crystals that are frequently identified in SF of OA patients, could be involved in the translocation of STING from ER to the Golgi via vesicles.[37] Subsequently, other mechanisms involved in the pathogenesis of CPP-IA or OA, such as crystal-induced cell damage, senescence, apoptosis, or shedding of microvesicles could lead to the leakage of STING from the cells.

We also demonstrated high concentrations of STING in SF of patients with OA, even in presence of low-grade inflammation. These findings corroborate a previous study by Guo *et al.*,[38] who reported an increased expression of STING in OA human and mice cartilage, where it promotes senescence and apoptosis in chondrocytes. In addition, in OA samples, we did not detect significant mRNA levels of Nrf2, a STING negative regulator. Indeed, it has been shown that increased expression of Nrf2 represses STING mRNA and protein levels in human cells,[2] thus suggesting that the high levels of STING in OA observed in our results could be due to the lack of regulation by Nrf2.

However, different from what was expected, an inverse correlation between STING and Nrf2 in inflammatory SFs was not found. This might be because other mechanisms may be involved in the pathogenesis of the diseases considered. For instance, although Nrf2 is recognized as a pivotal mediator in protecting against oxidative stress, it has been reported that it is necessary for the activation of the NLRP3 inflammasome and the IL-1β production induced by MSU and other types of microcrystals.[39,40] This could explain the high Nrf2 mRNA levels in gout and CCP-IA.

Another study shows that, although Nrf2 maintains the cellular defense against oxidative stress in RA, it is expressed and active in synovial membrane of patients with this disease.[41] In our results, Nrf2 mRNA levels were similar in gout, CPP-IA, and RA, but those of STING were significantly lower in RA, thus revealing a reduction in this inflammatory condition.

In conclusion, our study highlights the expression of STING in SF of patients affected by the most common joint diseases. Further investigations are warranted to ascertain the role of STING in articular and bone tissues homeostasis and the possible presence of exogenous and damaged DNA/RNA in SF. Although the exact role of STING remains unknown, our results suggest that the c-GAS-STING pathway may be involved in the pathogenesis and progression of arthropathies.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; AS, RL, and RR contributed to the conception and experimental design of the study; AS, PG, and FO performed the biological experiments, analyzed the data, and prepared the figures; AO and ML collected the biological samples and revised the manuscript for important intellectual content; AS, RL, and PG wrote and edited the manuscript; and AD and RR critically revised the manuscript. All authors read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The study procedures were approved by the Institutional Review Board of the University of Padua (Prot. N. 3304/AO/14). All patients signed a written consent form to participate in this study.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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