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# Systemic activation of NLRP3 inflammasome in patients with severe primary Sjögren's syndrome fueled by inflammagenic DNA accumulations

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ABSTRACT

Sjögren's syndrome (SS) patients manifest high cell-free DNA (cf-DNA) levels in serum, associated with impaired DNaseI activity. Undegraded DNA may accumulate in tissues and act as an inflammasome-activating signal. Herein, we investigated the occurrence of aberrant DNA build-up in various biologic compartments of SS patients and its correlation with the activity of NLRP3 and AIM2 inflammasomes. For this purpose, we evaluated sera, PBMC, circulating monocytes and salivary glands (SG) from different SS patient subgroups and controls. We found that SS patients at high risk for lymphoma and those with established lymphoma display high serum cf-DNA levels, substantial extranuclear DNA accumulations in PBMC and SG tissues, a unique NLRP3 inflammasome gene signature in PBMC, and significantly increased serum IL-18 and ASC levels. In these patients, the circulating monocytes manifested NLRP3 inflammasome activation and increased response to NLRP3 stimuli, whereas SG-infiltrating macrophages exhibited signs of NLRP3 activation and pyroptosis. Cell-free nucleic acids isolated from patients' sera competently primed the activation of both NLRP3 and AIM2 inflammasomes in healthy monocytes. SS patients also manifested diminished DNaseI activity in serum and DNaseII expression in PBMC, which inversely correlated with indices of inflammasome activation. DNaseII gene-silencing in healthy monocytes led to cytoplasmic DNA deposition and activation of inflammasome-related genes and of caspase1. Our data reveal the occurrence of systemic NLRP3 inflammasome activation in severe SS, which is associated with widespread extranuclear accumulations of inflammagenic DNA and impaired DNA degradation. These findings can provide novel biomarkers and new therapeutic targets for the management of SS patients with adverse outcomes.



## 1. Introduction

Primary Sjögren's syndrome (SS) is a relatively common autoimmune disorder that is primarily characterized by chronic lymphoepithelial inflammation in the exocrine glands, mainly the salivary and lachrymal glands [1]. SS may extend from disease confined to the exocrine glands (organ-specific exocrinopathy) to various extraglandular manifestations (systemic disease), as well as the development of non-Hodgkin's lymphoma (usually of mucosa-associated lymphoid tissue type; MALT-NHL) in 5% of patients [1]. Approximately one-third of patients manifest clinical and laboratory features that represent high-risk prognostic factors for

*Abbreviations:* SS-LR, SS patients with low risk for lymphoma development; SS-HR, SS patients with high risk for lymphoma development; SS-Ly, SS patients complicated with mucosa-associated non-Hodgkin's lymphoma (MALT-NHL); HC, healthy controls; SG, salivary gland; DNaseI, deoxyribonuclease-I; DNaseII, deoxyribonuclease-II; TREX1, Three Prime Repair Exonuclease-1; NLRP3, NACHT, LRR and PYD domains-containing protein 3 (NALP3); AIM2, absent in melanoma 2; PYCARD, PYD and CARD Domain Containing protein; ASC, Apoptosis-associated speck-like protein; ESSDAI, EULAR Sjögren's syndrome disease activity index; cf-DNA, cell-free DNA; cf-NA, cell-free nucleic acids; NIG, nigericin.

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lymphoma development (SS with high risk; SS-HR), as opposed to those without such adverse prognostic factors (SS with low risk; SS-LR) [1]. Although the aetiopathogenesis of the disease remains unclear, evidence indicates the contribution of both innate and adaptive immunity in inflammatory reactions [2–4].

Inflammasomes are a group of multimeric protein complexes that represent a major innate immune response platform, whose activation leads to the production of the pro-inflammatory cytokines IL-1β and IL-18 [5]. Previous experimental evidence from our laboratory and others had indicated that the inflammatory processes in the salivary lesions and the peripheral blood of SS patients involve several aberrations that can be attributed to the activation of the inflammasome, including the high production of IL-1β and IL-18 cutokines and the activation of P2X7R-inflammasome axis [6–11]. In addition, the involvement of NLRP3 inflammasome in SS exocrinopathy has been indirectly implied by studies in patients and animal models suffering from dry eye disease [12]. Nevertheless, the activation status of inflammasomes in the various biologic compartments of SS patients, as well as the putative triggering mechanisms, have not been previously addressed.

Various pathogen-derived and endogenous molecules, including nucleic acids that are released following tissue injury [13], have been shown to induce inflammasome activation. The inflammagenic accumulation of DNA is normally prevented by deoxyribonucleases acting in the serum (DNaseI), the cytosol (TREX1/DNaseIII) and the lysosomes (DNaseII). Recent studies from our laboratory have indicated that SS patients manifest aberrant clearance of apoptotic cells by phagocytes and impaired serum DNaseI-mediated degradation of necrotic cell remnants, which are associated with the occurrence of increased amounts of circulating nucleosomes and cell-free DNA (cf-DNA) in the blood [14,15]. On this basis, we herein sought to investigate the status of inflammasome activation in sera, PBMC, circulating monocytes and minor salivary gland specimens derived from SS patients belonging to various disease severity subgroups, including SS-LR and SS-HR patients, as well as SS patients who had already developed MALT-NHL (SS-Ly). In these patients, the relationship of inflammasome activation with the aberrant presence of circulating undigested DNA was also studied. The comparative gene and protein expression analyses of patients and controls revealed that the peripheral monocytes and the salivary gland-infiltrating macrophages of SS patients manifest activation of NLRP3 inflammasome, which is particularly evident among patients with severe disease. Our data also indicated that such activation is most likely induced by widespread accumulations of extranuclear DNA in the serum, cells and tissues of patients, wherein the deficient DNA degradation by deoxyribonucleases (DNaseI and DNaseII) appears to play a major role.

## 2. Materials and methods

### 2.1. Reagents

A detailed description is provided given in Supplementary Materials and Methods.

### 2.2. Patients and samples

Experiments involved specimens from 76 SS patients (70 women and 6 men, median age: 59 years, range: 15–83 years), who were diagnosed according to published criteria [16], 11 non-SS disease controls (all women, median age: 56 years, range: 20–81 years, who had no histopathologic or serologic evidence for SS) and 30 age-matched healthy controls (HC, all women, median age: 58 years, range: 18–75 years). SS patients studied included 24 with high-risk predictors for lymphoma development (SS-HR) [17], 16 without these adverse predictors (low-risk for lymphoma, SS-LR) and 36 with established MALT-NHL lymphoma (SS-Ly). The detailed demographics, clinical and laboratory features of SS patients studied are shown in Supplementary Table 1. All individuals were assessed and followed at the Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Greece. The study was approved by the Ethics Committee of the School of Medicine, National University of Athens (No-5107). Details on the collection and handling of specimens from peripheral blood and minor salivary gland tissues are presented in Supplementary Materials and Methods.

### 2.3. Isolation of cytosolic fractions from PBMC and measurement of DNaseII activity in protein extracts

Cytosolic extracts from $3 \times 10^6$ PBMC were prepared using a commercial Nuclear Extraction Kit (Cayman Chemical) and protein concentration was determined by the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific) according to manufacturers' instructions. DNaseII activity in cytosolic extracts was assessed by single-radial enzyme diffusion (SRED) assay, using a modified previous protocol described in Supplementary Materials and Methods [18].

### 2.4. Measurement of DNaseI activity and quantification of cf-DNA in the sera

Serum DNaseI activity was assessed by the SRED assay, as previously [14]. Nucleic acids were extracted from sera using the QIAamp DSP Virus Kit (Qiagen) and the levels of cf-DNA were determined by RT-PCR, as previously [14].

### 2.5. Real time quantitative polymerase chain reaction (RT–PCR)

Real-time quantitative PCR Assays were performed for the evaluation of inflammasome-related genes, MX1 and nuclease-encoding genes. Protocols and primer-sets are shown in Supplementary Materials and Methods.

### 2.6. Calculation of cumulative NLRP3 score

In each study subject, the standardized expressions of the NLRP3 inflammasome-related genes NLRP3, ASC/PYCARD, procaspase1, pro-IL-1β and pro-IL-18 were calculated on the basis of reciprocal gene expression in the HC group, as previously described for interferon-signature [19]. Accordingly, the standardized expressions of these 5 genes were subsequently summed to provide the cumulative NLRP3 expression score.

### 2.7. Immunofluorescence and confocal microscopy

Microtome sections obtained from formalin-fixed paraffin-embedded MSG specimens were subjected to citrate treatment for antigen retrieval as previously [20]. The protocols followed for confocal microscopy imaging and colocalization studies are described in detail in the Supplementary Materials and Methods.

### 2.8. Flow cytometry

The expression levels of active caspase1 were assessed by flow cytometry (FACS-Calibur flow cytometer and CellQuest software) in CD14-positive peripheral blood monocytes using the FAM-YVAD-FMK FLICA reagent. Further details are described in the Supplementary Materials and Methods.

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