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# Circulating Interferon-Inducible Protein IFI16 Correlates With Clinical and Serological Features in Rheumatoid Arthritis.

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#### RUNNING HEAD: IFI16 and clinical/serological features in RA

**TITLE:** The circulating interferon-inducible protein IFI16 correlates with clinical and serological features in rheumatoid arthritis.

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#### ABSTRACT

**Objective:** The IFN-inducible protein 16 (IFI16) has been detected in sera from patients with autoimmune/inflammatory diseases but not in healthy subjects. This leaking leads to loss of tolerance towards this self-protein and the development of autoantibodies. In this study, clinical significance of both IFI16 protein and anti-IFI16 antibodies (Abs) in rheumatoid arthritis (RA) was investigated.

**Methods:** IFI16 protein and anti-IFI16 Abs levels were assessed by ELISA in serum samples from 154 RA patients and 182 healthy controls (HC), and in synovial fluid (SF) samples from 21 RA patients and 25 patients with osteoarthritis (OA).

**Results:** Mean serum levels for both IFI16 and anti-IFI16 Abs were higher in RA than in HC with a direct correlation between IFI16 concentration and anti-IFI16 Abs titer. The majority of RA patients with detectable circulating IFI16 protein were also positive for RF/ACPA. The latter group was found positive for anti-IFI16 Abs as well. The mean SF concentrations of both IFI16 protein and anti-IFI16 Abs were higher in RA when compared with control OA. Interestingly, the presence of circulating IFI16 protein, but not anti-IFI16 Abs, significantly correlated with RA-associated pulmonary disease. This correlation was not dependent on the presence of anti-IFI16 Abs, gender and smoking habit.

**Conclusion:** Our data demonstrate that the high levels of circulating IFI16 in RA are more frequent in RF/ACPA-positive RA patients and significantly associated with pulmonary involvement. The relevance of circulating IFI16 protein as new clinical biomarker of RA should be verified with additional studies.

## SIGNIFICANCE AND INNOVATION

- IFI16 protein and anti-IFI16 autoantibodies are highly expressed in the serum and synovial fluid of patients with rheumatoid arthritis.
- Serum IFI16 protein is associated with pulmonary involvement and serological status (RF/ACPA) in rheumatoid arthritis patients.

#### **INTRODUCTION**

Type I interferons (IFNs) are early mediators of the innate immune response that potentiate the adaptive immune response through direct and indirect actions on dendritic cells (DCs), T and B cells, and natural killer cells. Increased expression of type I IFN stimulated genes (ISGs), namely the "type I IFN signature", has been described in a variety of autoimmune inflammatory disorders (1), including systemic lupus erythematosus (SLE) (2), primary Sjögren's syndrome (pSS) (3), systemic sclerosis (SSc) and rheumatoid arthritis (RA) (4).

As far as RA is concerned, IFNs have been suggested as pathogenic molecules due to their capability to drive a Th1 response (5) but, most intriguingly, the "type I IFN signature" has been reported as a marker of disease development and response to therapy. In fact, overexpression of some IFN-inducible genes in circulating immune cells has been associated with higher risk of developing RA in pre-symptomatic individuals, subjects with arthralgia and circulating rheumatoid factor (RF), and/or anti-cyclic citrullinated peptide antibodies (ACPA)(6). Moreover, a different baseline gene expression profile of type I ISGs has been associated with DAS28 or EULAR response outcome in RA patients treated with the anti-tumor necrosis factor (TNF) agents rituximab or tocilizumab(7-9). With this scenario in mind, it is intriguing to speculate that "type I IFN signature" may also be able to identify clusters of patients with different clinical picture and prognosis.

The nuclear phosphoprotein IFI16 is a member of the HIN200/Ifi200 gene family, whose overexpression drives early steps of an inflammatory response through NF-kB mediated secretion of proinflammatory molecules such as ICAM1, E-selectin, IL-8 and MCP-1(12, 14). The IFI16 protein is overexpressed in several systemic autoimmune diseases and its specific autoantibodies are prevalent in the sera of these patients, thus linking this autoantigen to the pathogenesis of autoimmune diseases (10,13,17). A number of data also indicate the disease

specific mislocalization of nuclear IFI16 in several inflammatory settings (11-12) while our group has recently demonstrated the occurrence of free IFI16 protein in the sera of autoimmune patients (16). Taken together, these observations suggest a role for IFI16 in inflammatory, detrimental cellular changes underlying autoimmune phenomena (12, 14).

Although in a previous study on 15 RA patients the authors failed to show increased levels of the IFI16 protein in patients' sera by comparison with normal controls (15), we have recently reported the occurrence of circulating IFI16 protein in patients' sera suffering from different systemic autoimmune disorders, including SSc, SLE, SS and RA (16), Interestingly, RA patients displayed the highest levels of circulating protein alongside with the highest percentage of subjects with IFI16 levels above the 95<sup>th</sup> percentile for controls.

Taken together, these observations prompted us to investigate the prevalence and clinical significance of the IFI16 protein and anti-IFI16 autoantibodies in both serum and synovial fluid from a large cohort of RA patients.

#### **PATIENTS AND METHODS**

Patients. One hundred and fifty four consecutive patients with RA according to the 1987 American College of Rheumatology criteria (18) were included in the study and 182 sex and age-matched healthy donors as controls (HC). At the time of enrollment, serum samples were collected and stored at -20°C until use. Twenty-one consecutive RA patients, that underwent arthrocentesis for knee arthritis and 25 patients with knee osteoarthritis (OA) requiring arthrocentesis were also included in the study for the evaluation of SF samples. Paired serum and SF samples were then obtained from 9 additional RA patients. Patients receiving intraarticular medications (e.g corticosteroids, hyaluronic acid) in the previous six months were excluded. SF samples were centrifuged and supernatants were stored at -20°C until use. Clinical and serological records were collected at the time of enrollment. Pulmonary involvement, defined as presence of interstitial lung disease (ILD), was ascertained with Xray and high resolution computerized tomography. This study was approved by the local Ethics Committee (CEAS) and written informed consent was obtained in accordance with the declaration of Helsinki.

Determination of antibody titers towards human recombinant IF116 by ELISA. Polystyrene micro-well plates (Nunc-Immuno MaxiSorp; Nunc, Roskilde, Denmark) were coated with a solution of recombinant IF116 in PBS. After blocking, sera were added in duplicate. After washing, horseradish peroxidase-conjugated rabbit anti-human IgG (Dako Cytomation, Carpinteria, CA, USA) was added. Following the addition of the substrate (TMB; KPL, Gaithersburg, MD, USA), absorbance was measured at 450 nm, using a microplate reader (SpectraCount, Packard, Packard BioScience Company). The background reactivity of the reference mixture was substracted to calculate the results. A standard curve was constructed by serially diluting IgG from an anti-IFI16 positive patient serum (13, 17).

Determination of extracellular IF116 by capture ELISA. For determination of circulating extracellular IF116 a capture ELISA was employed. Briefly, polystyrene micro-well plates (Nunc-Immuno MaxiSorp; Nunc, Roskilde, Denmark) were coated with a home-made polyclonal rabbit-anti-IF116 antibody (aa 478-729). Subsequently, the plates were washed and free binding sites were saturated with PBS/0.05% Tween/3% BSA. After blocking, sera were added in duplicate. Purified 6His-IF116 protein was used as standard. BSA served as negative control. The samples were washed and in each case monoclonal mouse anti-IF116 antibody (Santa Cruz, sc-8023) was added and incubated for 1h at room temperature. After washing, horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare Europe GmbH, Milan, Italy) was added. Following the addition of the substrate (TMB; KPL, Gaithersburg, MD, USA), absorbance was measured at 450 nm, using a microplate reader (SpectraCount, Packard, Packard BioScience Company). The determination of the concentration was carried out using a standard curve for which increasing concentrations of purified 6His-IF116 were used (16).

*Statistical analysis* All data analysis was performed using IBM-SPSS version 13.0 (IBM Corp. Armonk, NY, USA). As detailed throughout the manuscript, Chi square test, Mann Whitney U test or Kruskal-Wallis test with Dunn's test for multiple comparison post-hoc were employed to compare groups. Univariate and multivariate binary logistic regression were employed to identify any association between IFI16 protein/anti-IFI16 antibodies and clinical, demographic or serological variables. The significance level was two sided and set at p<0.05.

#### RESULTS

#### Association of circulating IFI16 protein or anti-IFI16 antibodies with clinical features.

The presence and levels of the IFI16 protein and anti-IFI16 autoantibodies in serum samples from RA patients and healthy controls were assessed by ELISA (for statistical performance characteristics of the tests please refer to on line additional material). As shown in Figure 1, mean levels of both IFI16 and anti-IFI16 were higher in RA in comparison with HC. According to a cut-off value of 27 ng/ml for the IFI16 protein and 113 U/ml for anti-IFI16 antibody (established as the 95<sup>th</sup> percentile of the control population) (16, 17), circulating IFI16 protein was present in 56/154 (36.4%) RA patients and in 6/116 (5,2%) HC, while anti-IFI16 antibodies were observed in 32/154 (20.8%) RA patients and in 9/182 (4.9%) HC, confirming a significantly higher prevalence of both protein and autoantibodies in RA (both p < 0.0001) in comparison with HC. It is worthwhile to mention that among the 56 RA patients with circulating IFI16 protein, only 27% were also positive for anti-IFI16 antibodies. In contrast, among the 32 patients positive for anti-IFI16 autoantibodies, 47% were also positive for the IFI16 protein. Interestingly, while a broad but normal distribution of anti-IFI16 values was observed in both RA and HC, the majority of RA samples displayed an intriguing distribution of IFI16 levels, being in most of the cases either strongly positive or clearly negative. Nevertheless, a significant correlation between IFI16 concentration and anti-IFI16 antibody titers was observed in the RA patients (Spearman's rho=0.23; p=0.004).

When patients were grouped according to the presence or the absence of either IFI16 protein or anti-IFI16 antibodies (with the above mentioned respective cut-off), some correlations with the clinical features were identified (Table 1). A significantly higher proportion of male patients displayed anti-IFI16 antibodies in the absence of circulating IFI16 protein (p<0.0003), whereas no correlation with age, age at diagnosis and disease duration was observed. Interestingly, IFI16 protein, but not anti-IFI16 antibodies was closed associated with positivity for both RF and anti-CCP antibodies being only 3 out of 56 IFI16-positive patients negative for both. Binary logistic regression analysis confirmed the association between circulating IFI16 protein and FR/anti-CCP positivity with an odds ratio (OR) of 13 (95% confidence interval-CI- 4.4-39, p<0.0001). Interestingly, as far as anti-CCP titer was concerned, a significant association with IFI16 positivity (OR=1.01, 95% CI= 1.006-1.023, p<0.01) was observed. In addition, anti-nuclear antibodies (ANA) were more prevalent in IFI16-positive patients compared to those without detectable levels of the protein in the serum (39% vs 23%).

From a clinical point of view, the prevalence of both erosive disease and rheumatoid nodules was similar in the patients grouped according to either the presence or absence of IFI16 protein and anti-IFI16 Abs (Table 1). The disease activity score (DAS) 28, available at the time of blood collection for 90 patients, was correlated with neither circulating IFI16 nor anti-IFI16 antibody titers (p= 0.6 and 0.8 respectively).

Interestingly, we found that RA-associated pulmonary disease was significantly associated with the presence of circulating IFI16, but not with anti-IFI16 autoantibodies. Since this association was found in circulating IFI16-positive patients with or without anti-IFI16 antibodies, we performed a binary logistic regression in an attempt to quantify the association between the presence or absence of IFI16/anti-IFI16 antibodies and pulmonary involvement. The OR for pulmonary disease in IFI16-positive patients was 4 (95% confidence interval-CI=1.6-10.4; p≤0.003) and was not dependent on the presence of anti-IFI16 antibodies (OR=4; 95% CI=1.6-10.2; p≤0.004). When the same analysis was performed taking into account the protein concentration rather than presence/absence of circulating IFI16, it appeared that increasing amounts of the protein did not affect the OR for pulmonary

involvement (p=0.06). It is worthwhile to mention that male gender, smoking and ANA were also associated with pulmonary involvement in our study cohort (Table 2). However, a multivariate analysis, including the presence of circulating IFI16 protein, revealed that this variable was significantly associated with pulmonary involvement independently of gender, smoking habit and ANA positivity.

#### Both IFI16 protein and anti-IFI16 antibodies are also present in synovial fluid

As the main immunopathogenic process in RA takes place in the joints, we were interested to verify whether IF116 protein and/or anti-IF116 antibodies were also detectable in the SF from RA patients. As shown in Figure 2, the mean concentrations of both IF116 protein (A) and anti-IF116 antibodies (B) in SF were significantly higher in RA patients in comparison with the control OA patients, ( $p\leq0.05$  and p<0.01, respectively). In both groups, IF116 and anti-IF116 levels were not correlated in SF (p=0.5 and 0.6 respectively) and no association between SF total/differential cell count and levels of either IF116 or anti-IF116 antibodies was found. As shown in Figure 2C, the analysis of paired serum and SF samples in RA revealed a complete concordance in IF116 and anti-IF116 positivity or negativity in the two compartments, except one patient positive for IF116 in the serum, but not in the SF.

#### DISCUSSION

Compelling evidence supports the pathogenic role of IFNs in autoimmune inflammatory diseases that are characterized by the overexpression of a number of IFN-inducible genes, namely the "IFN signature" (1). Among the molecules encoded by IFN-inducible genes, the IFI16 protein has been recently found to be present in sera from patients with a number of autoimmune diseases (12). Among these disorders, the highest serum levels of the IFI16 protein were found in a limited cohort of RA subjects (16). In the present study, we confirm in a large cohort of RA patients that they display higher concentrations of circulating IFI16 protein when compared with HC. Moreover, in contrast to the negative data of a previous report, performed in a small number of RA patients and using a 1<sup>st</sup> generation ELISA (10), we have also found statistically significant higher levels of anti-IFI16 antibodies in RA patients in comparison with HC by a 2<sup>nd</sup> generation ELISA with HPLC-purified antigen (13).

The presence of circulating IFI16 protein in the serum of RA patients is an attractive finding that draws a number of considerations. IFI16 is a nuclear pathogen DNA sensor that can be mislocalized in the cytoplasm and secreted in the extracellular milieu in several inflammatory settings, including virus infections (11, 19). When exposed to the extracellular environment, IFI16 is supposed to interact with components of the innate immune response, thereby inducing pro-inflammatory molecules (16). In addition, IFI16 overexpression in endothelial cells is sufficient to induce apoptosis that is mediated by the simultaneous activation of caspase 2 and caspase 3 (14). Taken together, these findings support a role for the IFI16 protein in the initial steps of the inflammatory process underlying autoimmune disorders, as its overexpression, mislocalization and secretion lead to break-down of tolerance towards this self-protein and eventually anti-IFI16 antibodies development (11).

The immunological relationship between IFI16 autoantigen and its autoreactive antibodies in RA may be supported by a significant, even if modest, correlation found in the present study. However, it is worth mentioning that only a subgroup of RA patients with circulating IFI16 protein levels above the cut-off were also positive for anti-IFI16 antibodies while a substantial number of patients with low or undetectable circulating IFI16 levels displayed significant amounts of specific autoantibodies. The presence of both protein and autoantibodies in the peripheral blood may be the result of an immune response occurring in inflamed tissues. It may be also speculated that a recirculation of B cells from RA target organs into the bloodstream can promote their contact with tissue resident cell types that aberrantly display IFI16 protein. In RA, the more evident inflammatory processes take place in the synovium. Consistent with this assumption, our results show that SF from RA inflamed joints contains both the IFI16 protein and anti-IFI16 antibodies and their positivity is closed associated with that in the serum. Thus, IFI16-specific B cells may originate in the synovial microenvironment following the release of IFI16 by, for example, synovial fibroblasts, stimulating the local production of autoantibodies subsequently released in the circulation. However, also considering the lack of correlation between IFI16 and anti-IFI16 in SF and the demonstration of possible IFI16 positivity in the serum, but not in the SF, we cannot rule out that the IFI16-induced immune response may take place in other or additional sites important for RA pathogenesis and autoantibody generation, such as the lung. In this context, the variable presence of IFI16 protein and/or anti-IFI16 antibodies in the serum may reflect different amounts of free protein or autoantibodies released in the bloodstream, explaining the presence in some sera of anti-IFI16 with undetectable circulating protein. In addition, it may be also hypothesized that in patients with circulating IFI16 protein and lack of anti-IFI16 antibodies, they will appear in later stages of the disease.

According to our data, significant levels of circulating IFI16 protein were detected in about one third of our RA population, while anti-IFI16 autoantibodies were present in about 20% of the them. IFI16 protein release appears to be specifically associated with RA patients displaying circulating RF/anti-CCP antibodies. These findings deserve some comments: i) IFI16-induced inflammation in RA appears to be more relevant in patients developing autoimmune reactions against not only citrullinated proteins, but also nuclear autoantigens. It may be intriguing to verify if the few IFI16 positive patients without other detectable circulating autoantibodies display antibodies against carbamylated proteins that seem to represent an alternative RA biomarker with a possible pathogenic role independent of anti-CCP; ii) RA patients with circulating RF and, particularly, anti-CCP antibodies, are more prone to develop extra-articular manifestation of the disease, including pulmonary involvement (20); iii) the observed association between circulating IFI16 protein and lung manifestation, irrespective of the presence of anti-IFI16 antibodies, appears to fit well in the context of seropositive RA patient subset that displays very high risk of pulmonary disease (21); iv) the previously reported association between pulmonary involvement, male gender and smoking is also confirmed in this study (22), although circulating IFI16 protein was independently associated with lung involvement.

In the present study, anti-IFI16 antibodies, but not the IFI16 protein, were more prevalent in male gender. Previous reports indicate that anti-IFI16 antibodies may exert a protective rather than proinflammatory role in autoimmune disorders (10, 13, 17); if this can be considered as an attempt of the immune system to defend tissues against a stronger and more aggressive inflammatory process, as occurs in RA males, remains a matter of debate. Other intriguing questions, however, arise from the close relationship between gender, smoking and lung disease in RA from a pathogenic point of view. It has been hypothesized that the increased frequency of RA-related pulmonary manifestations in men may be due to more prevalent and

higher consumption of tobacco (22). Tobacco smoking, in fact, may precipitate site-specific citrullination in the lungs leading to the generation of anti-CCP antibodies (23). This process, that may occur very early in RA even before disease onset (24), appears to play a key role in triggering and perpetuating RA inflammation thus representing a pathogenic link with the proinflammatory role exerted by the IFI16 protein. The fact that RA lung involvement was associated with circulating IFI16 positivity, but didn't correlate with the serum levels of the protein, is not surprising since the great majority of IFI16-positive patients displayed very high levels of the protein. Accordingly, RA pulmonary manifestations are usually associated with very high titers of anti-CCP (22).

Altogether, the results of this study confirm the occurrence of the nuclear DNA sensor IFI16 protein in RA patients' sera. This autoantigen is more prevalent in subjects with circulating RF/anti-CCP antibodies and may mirror its involvement in inflammatory process in target tissues. Although IFI16 can be detected in inflamed joints, it does not seem to be associated with joint disease activity or erosions. Although the observed significant association with lung involvement suggests an important pathogenic role in the inflammatory processes characterizing RA, the clinical and prognostic significance of circulating IFI16 protein in this disorder needs to be clarified with larger and prospective studies.

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#### FIGURE LEGEND

**Figure 1** Expression of the IFI16 protein (A) and anti-IFI16 antibodies (B) in the serum of HC (n. 116 and n. 182, respectively) and RA patients (n. 154). Bars indicate mean values. Dotted lines mark cut-off levels of positivity as determined by the 95<sup>th</sup> percentile of the control population. P values were calculated with Mann Whitney U test.

**Figure 2** Expression of the IFI16 protein (A) and anti-IFI16 antibodies (B) in the synovial fluid of OA (n. 25) and RA patients (n. 21). C) Expression of the IFI16 protein and anti-IFI16 antibodies in 9 paired samples of serum and synovial fluid of RA patients. Bars indicate mean values. Dotted lines mark cut-off levels of positivity as determined by the 95<sup>th</sup> percentile of the control population. P values were calculated with Mann-Whitney U test.

	All	IFI16 pos	IFI16 neg	р	anti-IFI16 pos	anti-IFI16 neg	р
Number of RA patients	154	56 (36)	98 (64)	na	32 (21)	122 (79)	na
Male	27 (17)	12 (21)	15 (15)	0.4	10 (31)	7 (6)	0.0003
Age	64±1	64±2	64±1	0.5	66±2	63±1	0.3
Age at diagnosis	52±1	53±2	51±1	0.9	53±2	51±1	0.9
Disease duration	13±0.7	12±1	14±0.9	0.7	14±2	13±0.8	0.9
Autoantibodies							
none	42 (27)	3 (5)	39 (40)	< 0.0001	5 (16)	37 (30)	0.1
RF+	19 (12)	7 (12)	12 (12)	0.8	5 (16)	14 (11)	0.5
anti-CCP+	9 (6)	1 (2)	8 (8)	0.1	2 (6)	7 (6)	0.7
RF+/anti CCP+	84 (55)	45 (81)	39 (40)	< 0.0001	20 (62)	64 (53)	0.3
ANA+	45 (29)	22 (39)	23 (23)	0.04	13 (41)	32 (26)	0.1
Erosions	83 (54)	35 (62)	48 (49)	0.1	14 (44)	69 (56)	0.2
Nodules	10 (6)	5 (9)	5 (5)	0.5	4 (12)	5 (4)	0.08
Pulmonary involvement	23 (15)	15 (27)	8 (8)	0.004	6 (19)	17 (14)	0.6
RF, rheumatoid factor, CCP, cyclic citrullinated peptide, ANA, anti-nuclear antibodies, pos, positive, neg							
negative, na, not applicable							

# **Table 1:** Demographic, clinical and serological characteristics of RA patients

**Table 2.** Univariate and multivariate binary logistic regression analysis of factors associated to the presence of RA pulmonary involvement

		Univaria	te	Multivariate		
Variable	OR	95% CI	p value	OR	95% CI	p value
Male gender	4	1.5-10.6	0.005	2.7	0.9-8.3	0.08
Smoking*	2.8	1.1-7.5	0.04	1.6	0.5-4.9	0.4
ANA	2.6	1.04-6.4	0.04	2.07	0.7-5.8	0.2
IFI-16	4	1.6-10.4	0.003	3.1	1.1-8.7	0.03
OR = odds ratio; CI = confidence interval. *current and past smokers are cumulated						

## Figure 1



Figure 2



# **Supplementary Material**

**Table 3.** Statistical performance characteristics of Anti-IFI16 and IFI16 assessment in rheumatoid arthritis.

	Anti-IFI16 autoantibodies	IFI16 protein
Sensitivity	20.78%	36.30%
Specificity	95.05%	94.80%
Positive predictive value	78.05%	90.30%
Negative predictive value	58.64%	52.88%