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Identification of *Prevotella Oralis* as a Possible Target Antigen in Children with Enthesitis related arthritis

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Abstract

Objectives: Patients with Crohn Disease often produce antibodies against flagellated intestinal bacteria. There are mixed data as to whether such antibodies are present in patients with spondyloarthritis. Our objectives were to evaluate for the presence of antibodies against intestinal organisms in children with enthesitis related arthritis (ERA).

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Declaration of interests

None

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Methods: Children with ERA and healthy controls were recruited at three sites. Sera were plated on a nitrocellulose array and incubated with labelled antibodies to human IgA and IgG.

Results: At UAB, patients and controls had similar antibody levels against the majority of the bacteria selected, with the exception of increased IgA antibodies among ERA patients against *Prevotella oralis* (1231 [IQR 750, 2566] versus 706 [IQR 428, 1106], p = 0.007.) These findings were partially validated at a second but not at a third site.

Conclusions: ERA patients may produce increased IgA antibodies against *P. oralis.* The possible significance of this finding bears further exploration.

Keywords

Antibodies; Enthesitis-Related Arthritis; Immunoglobulin A; Prevotella; Spondyloarthritis

1. Introduction

The human intestine is colonized with an estimated 100 trillion bacteria, a process that begins shortly after birth [1]. Long ignored, it has become increasingly clear that these bacteria play important roles in immune function as well as in a variety of autoimmune and inflammatory disorders [2, 3]. Particular interest has been afforded the role of the microbiota in the related conditions of inflammatory bowel disease (IBD) and spondyloarthritis (SpA), with studies in both diseases demonstrating differences in the contents of the fecal microbiota, including increased or decreased relative abundance of organisms such as *Faecalibacterium prausnitzii* [4, 5], *Ruminococcus gnavus* [6, 7], and the *Bacteroides* genus, including *B. fragilis* [8, 9]. Patients with IBD also have dysregulated response to enteric antigens. Specifically, through use of a phage display library, our group previously identified bacterial flagellins as important antigenic targets in patients with IBD [10]. Although the work was conducted in mice, anti-flagellin antibodies are also present in about 50% of patients with Crohn Disease [10], where they serve as markers of poor prognosis [11]. There are mixed data as to whether these antibodies are present in patients with SpA, with some [12, 13] but not all [14] studies showing elevated levels of IBD-associated antibodies in this population. There are no pediatric data, however.

Our group developed an antigen array system which we have used to screen patients for IgG and IgA antibodies against enteric antigens [15]. To evaluate for the presence of antibodies against enteric antigens, we applied this array with serum from children with enthesitis related arthritis (ERA). To our surprise, we found that while flagellin reactivity among these subjects was not elevated, they did demonstrate increased IgA reactivity against *Prevotella oralis.*

2. Methods

2.1 Patients

This study was conducted at three sites: Children’s of Alabama / University of Alabama at Birmingham (UAB), Cincinnati Children’s Hospital Medical Center (CCHMC), and Boston Children’s Hospital (BCH). The derivation of the CCHMC cohort was described previously

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and included treatment-naïve subjects recruited at CCHMC (n = 9), the Children's Hospital of Philadelphia (n = 10), St. Vincent's Hospital in Indianapolis (n = 3), and one each from the North Shore / Long Island Jewish Medical Center and Toledo Children's Hospital [16], Patients meeting the International League of Associations for Rheumatology criteria for ERA [17] were enrolled, modified to allow inclusion of children with a family (but not personal) history of psoriasis. Children with IBD were excluded from the initial arrays. Healthy controls at UAB and BCH consisted of children referred to our clinics to evaluate for arthritis, but found to have a non-inflammatory cause of joint pain; additional controls at UAB were recruited from the general population through advertisements or through relatives affiliated with the hospital. Healthy control recruitment at CCHMC took place through the Cincinnati Children's Genomic Control Cohort, which is a population representative sample of 1020 children age 3 – 18 years from the 7 counties that comprise the Greater Cincinnati Region. Controls for the current study were selected so as to ensure matching based upon age at serum collection, sex, and race. Informed consent was obtained from all guardians, and assent / consent were obtained from pediatric patients as per institutional guidelines. Institutional Review Board approval at the UAB as well as at the satellite sites was obtained (UAB IRB approval # IRB-130507003.)

2.2 Antigen array

The array was performed as described [15]. Briefly, proteins, including flagellin antigens [18] as well as bacterial lysates and tetanus toxoid as a control antigen, were diluted in TRIS buffer (pH 8.0) with 0.5% SDS at 0.2 mg/mL and printed onto FAST slides (Global Life Science Solutions, Sanford, ME) using an Array IT robot (Sunnyvale, CA) in quadruplicate. Slides were air-dried overnight, blocked with Super Block (Thermo Scientific, Rockford, IL), and probed with human sera at 1:100 dilution for one hour. After washing, slides were incubated with DyLight 650-labelled Donkey anti-human IgG (Invitrogen, Carlsbad, CA) and DyLight 550-labelled goat anti-human IgA (Immuno Reagents, Raleigh NC) and read on an Axon GenePix 4000B dual laser microarray reader (Molecular Devices, Sunnyvale, CA). The peptides and whole bacterial lysates included on the arrays are shown in Tables 1 and 2, respectively. Preparation of the outer membrane (OM) proteins was performed largely as described [19], except for use of sonication (three 30-second pulses at 70%) instead of French press. Preparation of the extracellular polysaccharide was performed as described [20].

2.3 Statistical analysis

Comparisons between dichotomous variables were performed with the Mann-Whitney test (two groups) or Kruskal-Wallis test (three groups), while comparison of continuous variables was performed with the Spearman correlation coefficient.. Correction for multiple comparisons was performed with the Benjamini-Hochberg test. Analyses were performed with R, version 3.6.3.

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3. Results

3.1 Patients

Clinical and demographic features of the subjects are shown in Table 3. By design, patients and controls recruited through CCHMC were well-matched by age and sex, while at BCH and UAB, controls were slightly older and slightly younger, respectively, than the ERA patients. All of the subjects at BCH and CCHMC were naïve to immunosuppressive therapy, while the UAB subjects were heterogeneous with respect to prior therapies.

3.2 Antigen array studies

Antigens present on the array are shown in Tables 1 and 2. As shown, UAB patients and controls had similar levels of IgA (Figure 1A) and IgG (Figure 2A) antibody production against the majority of the bacteria selected, the only exception being *Prevotella oralis.* Clear separation of the groups is observed, with ERA patients demonstrating increasing net fluorescent intensity of 1231 (IQR 750, 2566) to 706 (IQR 428, 1106) ($p = 0.007$), albeit the finding was non-significant at 0.161 when corrected with the Benjamini-Hochberg test. No other comparisons achieved even an uncorrected statistical significance. Among the children with ERA, there were no associations between *P. oralis* reactivity and age of serum collection, sex, HLA-B27 status, use of immunosuppressive therapy, ESR, or CRP. For example, male patients demonstrated reactivity of 1243 (IQR 803, 2566) as compared to 1167 (IQR 750, 2282) for female patients ($p = 0.914$), and if anything, patients previously exposed to immunosuppressive therapy may have had increased reactivity (1702, IQR 915, 3075) compared to 952 (IQR 659, 1804) for treatment-naïve patients ($p = 0.091$). With respect to IgG reactivity, ERA patients demonstrated a non-significant increase in reactivity against *P. oralis* of 1582 (IQR 736, 2485) compared to 776 (IQR 538, 1656), $p = 0.067$; with nominally significant decreases in reactivity against *P. copri* (418 [IQR 255, 624] versus 622 [434, 858], $p = 0.005$) and *P. intermedia* (7104 [IQR 5086, 10748] versus 12016 [IQR 7956, 15172], $p = 0.007$), although none of these differences withstood correction for multiple comparisons.

To validate our findings, serum samples were obtained from two additional sites: CCHMC and BCH. Given the geographic distance between all three sites, it is highly unlikely that any single subject was enrolled more than once; indeed, a thorough review of the medical records of the UAB patients did not identify any who had been treated at BCH or any of the sites that provided samples to CCHMC. Among the BCH patients, there appeared to be a subset demonstrating increased reactivity (1126 [IQR 453, 2657] for the patients versus 690 [IQR 505, 976] among the controls), albeit with an outlier among the controls (Figure 1C), the differences were statistically insignificant at $p = 0.113$. No IgA comparisons at BCH achieved nominal significance, while the only IgG comparison at BCH that did so was decreased reactivity against *Streptococcus pyogenes* among patients (1371 [IQR 921, 2352) as compared to controls (2008 [IQR 1720, 2594], $p = 0.035$. There were no differences in IgG reactivity among the subjects at BCH. At CCHMC, however, reactivity against *P. oralis* was virtually identical between the two groups. Decreased IgA reactivity against *Porphyromonas gingivalis* (806 [IQR 537, 1220) versus 1148 [833, 1612], $p = 0.050$) and *Rikenella microfusus* (250 [IQR 62, 404] versus 480 [IQR 320, 682], $p = 0.014$) were

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observed; as with BCH, decreased reactivity was observed against *S. pyogenes* (1750 [IQR 1262, 2199] versus 2901 [IQR 1825, 3140], p = 0.006. Controls at all three sites had similar IgA titers against *P. oralis* (UAB 706 [IQR 428, 1106], BCH 690 [ICR 505, 976], CCHMC 740 [IQR 306, 1220), p = 0.976; while among the patients, the levels at CCHMC were lower than those at the other two sites (UAB 1231 [IQR 750, 2566], BCH 1126 [ICR 453, 2657], CCHMC 551 [IQR 307, 1289), p = 0.020. When data from the three sites were pooled, the only significant difference in IgA reactivity was against *P. oralis* (1031 [IQR 512, 2333] versus 693 [IQR 409, 1157), p = 0.010. Likewise, when data from the three IgG arrays were pooled, increased reactivity to *P. oralis* (1497 [IQR 684, 3014] versus 869 [IQR 634, 1691], p = 0.040), along with decreased reactivity to *Streptococcus pyogenes* (1728 [IQR 921, 2621] versus 2525 [IQR 1716, 2988], p = 0.002) and possibly *P. gingivalis* (1029 [IQR 751, 1302] versus 1186 [IQR 878, 1618], p = 0.047) emerged. As with UAB, there were no associations between sex and *P. oralis* titers among the subjects at BCH and CCHMC, nor among HLA-B27 status and IgA or IgG *P. oralis* titers at CCHMC; the low numbers of subjects tested for HLA-B27 among the BCH cohort precluded analysis. As BCH and CCHMC both consisted of inception cohorts, it was not possible to include treatment status as a variable. Small associations between age at serum collection and both IgA (r = 0.275, p = 0.013) and IgG (r = 0.217, p = 0.052) *P. oralis* titers among the cohort as a whole were observed.

Flagellin antibodies associated with Crohn Disease [10, 11] were included on the array. Among the ERA patients at UAB, there were no differences in IgA or IgG reactivity against any of these antigens, nor among children in the CCHMC or BCH cohorts (Figures 1, 2.) We ran a repeat array that included 8 pediatric patients with Crohn Disease and 30 healthy controls recruited at UAB. Here, as expected, there was substantially increased IgG reactivity to Fla2 among the Crohn Disease patients (192 [IQR 147, 344) versus the controls (30 [IQR 5.9, 64]), p < 0.001), with otherwise similar IgG reactivity to the remainder of the antigens (not shown).

4. Discussion

We screened three cohorts of children with ERA for IgA and IgG antibodies against a panel of oral and intestinal bacteria. Our results suggested that at UAB and possibly at BCH, children with ERA demonstrated increased IgA antibodies against a single organism, *P. oralis*, as compared to healthy control subjects. In addition, pooled together, the subjects had increased IgA as well as IgG antibodies against *P. oralis*, although the differences in IgG reactivity were not significant at any of the sites. These differences were largely specific to *P. oralis*.

Important caveats here are the cross-sectional nature of the study and its small sample size, which impacted statistical power and underscored the exploratory nature of these findings. Furthermore, the demographics of the patients and controls at BCH and UAB were dissimilar with respect to age of the patients versus controls. It appears unlikely that this would account for our findings; the ERA patients were older than the controls at UAB and younger than the controls at BCH. The CCHMC cohort was not matched geographically, as the ERA subjects were derived from several sites, while the controls were all local; however,

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limiting the analysis to the subjects recruited at CCHMC did not alter the results (data now shown). It is puzzling that the differences in IgA and IgG reactivity were observed against whole *P. oralis*, but not against its outer membrane. We suspect this reflects limited statistical power and the presence of outliers among the controls, as visual inspection of the graphs (Figures 1 and 2) demonstrates some clustering with both the UAB and BCH cohorts for both IgA and IgG.

It bears mention that the presence of IgA antigenic targets does not necessarily indicate that either these targets or the antibodies directed against them are pathogenic; indeed, SpA has developed in patients lacking B cells completely [21]. However, such antibodies are a marker of class-switching and a mature response, thus potentially indicating T cell involvement. Although CD8 T cells are not required for disease in the HLA-B27 transgenic rat model [22], CD4 T cells are essential [23]. If this putative SpA-associated antigen is indeed targeted by CD4 T cells, then the basis for the association of HLA-B27 with SpA remains elusive, leaving our findings compatible with theories involving HLA-B27 protein misfolding as a basis for the unfolded protein response [24, 25]. It thus bears emphasis that among patients with ERA there was no association at UAB or CCHMC or among the group as a whole between IgA or IgG reactivity against *P. oralis* and HLA-B27 status.

Given the possibility of an antigenic trigger and the well-recognized association of sub-clinical ileitis in adult [26] and pediatric [27, 28] SpA, it is not implausible that such as trigger may be found within the gastrointestinal microbiota. The intestinal microbiota is the largest mass of adjuvant to which our immune system is exposed, and has profound lifelong effects on the maturation of our immune system [29]. Furthermore, the microbiota is clearly a trigger in IBD, in which several flagellin antigens are present in a large percentage of Crohn Disease patients, serving as markers of poor prognosis [10, 11, 18]. Additional supporting evidence for the role of the microbiota in SpA comes from animal studies showing abrogation of the disease in the germ-free state [30], as well as multiple studies in humans showing altered microbiota [8, 31–34].

Although our studies cannot exclude the possibility that antibodies to *P. oralis* represent a consequence of the disease process, the organism is an oral commensal that is scarcely present in the stool of children with ERA, some of whom were included in our previous studies on the fecal microbiota [4, 8]. Thus, elevated IgA antibodies against this organism in some ERA patients do not appear to represent a response to organisms present in an inflamed intestinal milieu. However, our data do not permit speculation as to the mechanism underlying this potential finding, nor the significance of the more robust association with IgA versus IgG antibodies. This latter finding is not unusual, as several immunologic conditions involving the gut are associated with stronger IgA responses to specific antigens, such as the tissue transglutaminase antibody in celiac disease [35] and the anti-Saccharomyces cerevisiae and outer membrane protein C antibodies associated with Crohn Disease [36].

In conclusion, our data suggest that oral and intestinal organisms such as *P. oralis* may be targeted in children with ERA. The possible significance of this finding bears further exploration.

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Abbreviations

BCH	Boston Children's Hospital
CCHMC	Cincinnati Children's Hospital Medical Center
ERA	enthesitis related arthritis
IBD	inflammatory bowel diseases
OM	outer membrane
SpA	spondyloarthritis
UAB	University of Alabama at Birmingham

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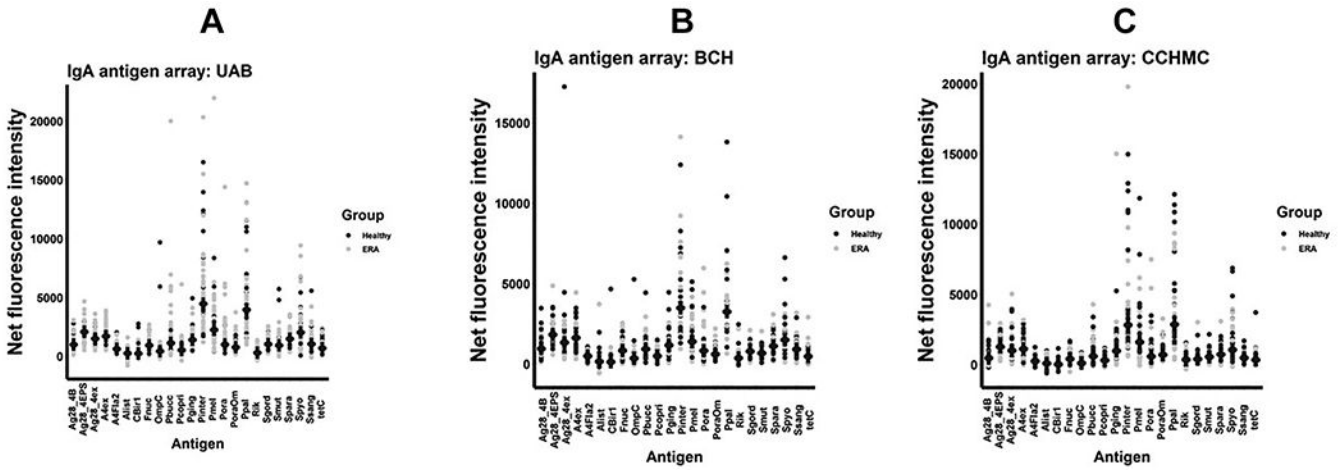

Figure 1.

IgA antigen arrays. Net fluorescent intensities (mean, SD) are shown for the IgA antigen arrays performed on serum obtained from subjects at the University of Alabama at Birmingham (A), Boston Children's Hospital (B), and the Cincinnati Children's Hospital Medical Center (C).

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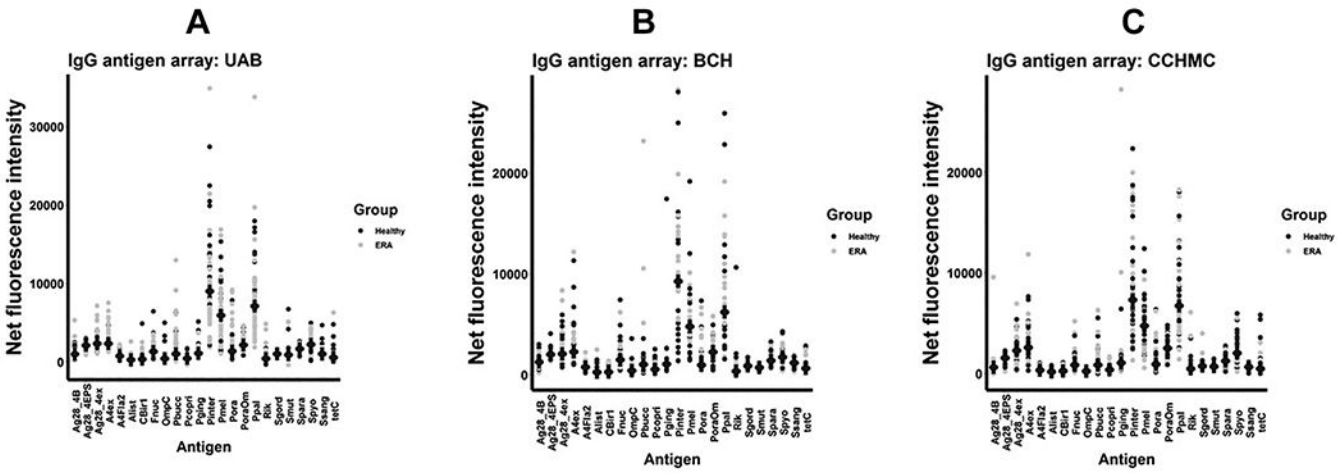

Figure 2.

IgG antigen arrays. Net fluorescent intensities (mean, SD) are shown for the IgG antigen arrays performed on serum obtained from subjects at the University of Alabama at Birmingham (A), Boston Children's Hospital (B), and the Cincinnati Children's Hospital Medical Center (C).

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Table 1.

Peptide antigens represented on the microarray

Name	Protein ID	Function	Primary species
28_4b	N/A	Unknown	Unspecified *Lachnospiraceae*
A4 Fla2	Flagellin	Motility	Unspecified *Lachnospiraceae*
CBir1	Flagellin	Motility	Unspecified *Lachnospiraceae*
OmpC	OMP from *E. coli*	Porin or transport	*Escherichia coli*
Tetc	Tetanus toxoid	Pathogenicity	*Clostridium tetani*

The source of the cloned antigens was described previously [15]. 28–4b is a 10 kDa band isolated using gel separation chromatography from the OM prep of 28–4 [18]. Abbreviations: OMP, outer membrane protein

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Table 2.

Bacteria represented on the array

Species	Phylum	Source
28–4	Firmicutes	Murine cecum [18]
28–4 EPS	Firmicutes	Murine cecum [18]
A4	Firmicutes	Murine cecum [18]
Alistipes putredinis	Bacteroidetes	ATCC 29800
Fusobacterium nucleatum	Fusobacter	Gifted from Hui Wu
Prevotella buccalis	Bacteroidetes	ATCC 35310
Prevotella copri	Bacteroidetes	DSMZ 18205
Porphyromonas gingivalis	Bacteroidetes	ATCC 33277
Prevotella intermedia	Bacteroidetes	ATCC 25611
Prevotella melaninogenica	Bacteroidetes	ATCC 25845
Prevotella oralis	Bacteroidetes	ATCC 33269
P. oralis outer membrane	Bacteroidetes	Prepared from ATCC 33269
Prevotella pallens	Bacteroidetes	ATCC 700821
Rikenella microfusus	Bacteroidetes	ATCC 29728
Streptococcus gordonii	Firmicutes	Gifted from Hui Wu
Streptococcus mutans	Firmicutes	Gifted from Hui Wu
Streptococcus parasanguinis	Firmicutes	Gifted from Hui Wu
Streptococcus pyogenes	Firmicutes	Gifted from Hui Wu
Streptococcus sanguinis	Firmicutes	Gifted from Hui Wu

Abbreviations: ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; EPS, extracellular polysaccharide.

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Table 3.

Subjects included in the array.

Feature	Controls	ERA
Children's of Alabama		
N	20	40
Age at diagnosis (years ±SD)	Not applicable	12 ± 3.2
Age at collection (years ±SD)	10.5 ± 4.9	13.2 ± 2.9
Males	11 (55%)	20 (50%)
Race		
Caucasian	16 (80%)	29 (72%)
African-American	2 (10%)	11 (28%)
Other	2 (10%)	0 (0%)
HLA-B27+	Not measured	12 / 37 (32%)
Medicines		
None	20 (100%)	17 (42%)
Conventional DMARDs alone		6 (15%)
TNFi ± conventional DMARD		17 (42%)
Boston Children's Hospital		
N	18	17
Age at diagnosis (years ±SD)	Notapplicable	11.2 ± 3.4
Age at collection (years ±SD)	14.5 ± 3.6	12.5 ± 3.7
Males	6 (33%)	11 (65%)
Race		
Caucasian	13 (72%)	14 (82%)
African-American	2 (11%)	1 (5.9%)
Other	3 (17%)	2 (12%)
HLA-B27+	1/1 (100%)	3/6 (50%)
Medicines	None	None
Cincinnati Children's Hospital Medical Center		
N	24	24
Age at diagnosis (years ± SD)	Not applicable	12.1 ± 2.7
Age at collection (years ± SD)	13.8 ± 3.0	14.0 ± 3.2
Males	18 (75%)	18 (75%)
Race		
Caucasian	23 (96%)	22 (92%)
African-American	1 (4.2%)	0
Other		2 (8.4%)
HLA-B27+	Not available	10 / 15 (67%)
Medicines	None	None

Abbreviations: DMARD, disease-modifying anti-rheumatic drugs; TNFi, tumor necrosis factor inhibitor