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Antibodies against three novel peptides in early axial spondyloarthritis patients from two independent cohorts

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Conflict of interests

Dana Quaden reports a research grant from the Agency for Innovation by Science and Technology Flanders (IWT) and a patent pending on the markers described in this manuscript. Patrick Vandormael reports a patent pending on the markers described in this manuscript. Pieter Ruytinx and Piet Geusens have nothing to disclose. Kristoff Corten reports consultancies/speaking fees and/or honoraria of more than $10,000 per year from DePuy Synthes and MedEnvision, stock ownership or options at MedEnvision, patents/licenses/licensing fees received or pending from DePuy Synthes and MedEnvision and royalties from DePuy Synthes. Johan Vanhoof, Jori Liesenborghs, Frank Van Reeth, Anouk Agten and Frank Vandenabeele have nothing to disclose. Kurt de Vlam reports consultancies/speaking fees and/or honoraria of less than $10,000 per year from Eli Lilly, Celgene, Galapagos, Johnson&Johnson and Novartis and a patent pending on the markers described in this manuscript. Veerle Somers reports a patent pending on the markers described in this manuscript.

ABSTRACT

Objective. The aim of this study was to identify novel autoantibodies in axial spondyloarthritis (axSpA) and determine their diagnostic potential in early axSpA patients and controls from two independent cohorts.

Methods. An axSpA cDNA phage display library was used to screen for novel immunoglobulin G (IgG) antibodies in plasma of early axSpA patients. Presence of these antibodies against novel Hasselt University (UH)-axSpA peptides was determined in 76 early axSpA patients, 75 non-specific chronic low back pain (CLBP) controls, 60 rheumatoid arthritis (RA) patients and 94 healthy controls (HC) from the UH cohort using enzyme-linked immunosorbent assays (ELISA). Antibody reactivity was further validated in 174 axSpA patients from the Leuven Spondyloarthritis (Biologics) cohort ((Bio)SPAR), including 79 early axSpA patients.

Results. We identified antibodies to 9 novel UH-axSpA peptides, corresponding to randomly formed peptides and to a novel axSpA autoantigen, Double Homeobox protein 4 (DUX4). Antibodies to 3 UH-axSpA peptides with the highest positive likelihood ratio (LR+) were significantly more present in early axSpA patients from the UH and (Bio)SPAR cohorts (14.2% (22/155)) compared to CLBP (5% (4/75)), resulting in 95% specificity. The LR+ for confirming axSpA using antibodies to these 3 UH-axSpA peptides was 2.7, which is higher than the currently used laboratory marker C-reactive protein (CRP). Testing for antibodies to these 3 UH-axSpA peptides in CLBP increased post-test probability for axSpA from 79% to 91%.

Conclusion. Antibodies to 3 UH-axSpA peptides could provide a novel tool for the diagnosis of a subset of axSpA patients.

INTRODUCTION

Axial spondyloarthritis (axSpA) is a rheumatic disease mainly characterized by chronic inflammation of spinal and sacroiliac joints, which can also affect peripheral joints and entheses, but also extra-articular tissues, such as the eyes, intestines and skin [1]. AxSpA diagnosis is classically based on the combination of clinical symptoms and in the case of radiographic axSpA, unequivocal radiographic damage such as the presence of radiographic sacroiliitis, or in the case of non-radiographic axSpA based on inflammation detected by dedicated MRI sequences [2]. In the absence of diagnostic criteria, classification criteria developed by the ASAS group (Assessment in SpondyloArthritis International Society) are often applied for diagnostic purposes in patients with suspect of axial and peripheral spondyloarthritis. These criteria combine physical examination, presence of sacroiliitis on imaging and laboratory tests for human leukocyte antigen (HLA)-B27 and C-reactive protein (CRP) [3, 4]. However, these classification criteria are not intended for diagnostic purposes and consequently perform less well, with lower sensitivity and specificity, making it difficult to distinguish axSpA patients from persons with non-specific chronic low back pain (CLBP) at an early disease phase [5-7]. Therefore, for many patients, axSpA diagnosis may be challenging and is often delayed by several years after the occurrence of first clinical symptoms [8, 9], posing a problem for early treatment initiation. This underlines the importance of the discovery of new objective biomarkers to improve early axSpA diagnosis. Although involvement of the humoral immune response in axSpA has long been disregarded, antibodies against several microbial, inflammatory, structural and related rheumatic antigen targets have also been described in axSpA [10]. These antibodies still require further validation in different cohorts to establish their potential as diagnostic biomarkers for axSpA, and their pathologic and clinical relevance still remains to be studied.

The aim of the current study was to perform an unbiased screening to identify novel autoantibodies in early axSpA patients that might provide a novel tool for early axSpA diagnosis. To this end, we applied the antibody profiling technique serological antigen selection (SAS). In this approach, a cDNA phage display library, expressing the antigens from axSpA hip synovial tissue, was constructed and screened for reactivity with immunoglobulin G (IgG) antibodies in plasma of early axSpA patients. The diagnostic value of antibodies to novel Hasselt University (UH)-axSpA peptides was validated in axSpA patients and controls from two independent cohorts.

PATIENTS AND METHODS

Patients and controls

Synovial tissues were collected from 3 axSpA patients, one rheumatoid arthritis (RA) patient and one healthy person with a sports injury. At Hospital East-Limburg (ZOL), Belgium, the axSpA hip tissues were collected during total hip arthroplasty and knee tissue from the healthy person was collected during knee surgery of the sports injury. In addition, RA knee tissue was collected during knee surgery at Jessa Hospital, Belgium. Upon collection, synovial tissues were snap frozen in liquid nitrogen and stored at -80˚C.

Plasma or serum of axSpA patients was collected at the ReumaClinic in Genk (UH cohort) (n=86) and at the University Hospitals Leuven ((Bio)SPAR cohort) (n=174) [11, 12]. All axSpA patients from the UH cohort (n=86) and 79 patients from the (Bio)SPAR cohort had a maximum time of 5 years since diagnosis and were considered early patients. Plasma from patients with non-specific CLBP (n=75), RA (n=60) and from healthy controls (HC) (n=104) was collected in Hasselt and Genk.

All axSpA patients were diagnosed by their treating rheumatologist and classified according to the ASAS classification criteria [3]. Patients with RA fulfilled the 1987 American College

of Rheumatology criteria for RA [13] and CLBP was diagnosed according to the European guidelines for the management of non-specific CLBP [14].

This study was conducted in accordance with the Helsinki Declaration and was approved by the local Ethical Committees of Jessa Hospital, Hasselt University and Hospital East Limburg (B243201422699). Biological samples of patients and controls were (previously) collected in different studies, which were approved by the ethical committees of Jessa Hospital, Hasselt University and University Hospitals Leuven (B322201215165, B243201627373, B32220083429 and B32220084074). All patients and controls provided informed consent and all human biological material used in this publication was kindly provided by the University Biobank Limburg (UBiLim) and the Biobank of University Hospitals Leuven.

Clinical characteristics of groups of interest

Novel axSpA antibodies and their antigenic targets were identified by the SAS procedure. This method uses an axSpA cDNA phage display library to screen for antibodies in pooled plasma of early axSpA patients. This early axSpA SAS pool consisted of 10 axSpA patients from the UH cohort which did not receive biological therapy, with a mean age of 40.6 ± 11.8 years, a mean disease duration of 1.4 ± 0.5 years, 5 (50%) were male and 8 (80%) were HLA-B27 positive. In the SAS procedure, counter- selection was performed using the HC SAS pool, consisting of 10 HC plasma samples, which were age- and gender-matched to the axSpA pool (mean age of 40.6 ± 12.3 years and 5 (50%) were male).

Antibody reactivity towards identified, individual peptides was determined by phage enzyme-linked immunosorbent assays (ELISA), in additional plasma pools of early axSpA patients and HC in order to select the best peptide targets. To this end, plasma of 60 additional early axSpA patients from the UH cohort was pooled into 6 early axSpA plasma pools of 10 patients each, and plasma of 30 additional HC was pooled into 3 HC plasma pools of 10 HC

each. These selected 60 early axSpA patients had a mean age of 44.1 ± 13.3 years, a mean disease duration of 3.0 ± 1.6 years, 36 (60%) were male and 38 (63.2%) were HLA-B27 positive. The 30 selected HC had a mean age of 46.7 ± 18.5 years and 12 (40%) were male.

Antibody reactivity against the 9 selected peptides was further validated using phage ELISA in individual plasma samples of 76 early axSpA patients, 75 persons with CLBP, 60 RA patients and 94 HC from the UH cohort and in serum samples of 174 axSpA patients, including 79 early axSpA patients, from the (Bio)SPAR cohort. Clinical characteristics of these axSpA patients from the UH (n=76) and (Bio)SPAR cohort (n=174), which were used for this validation screening, are summarized in Table 1. In brief, selected axSpA patients from the UH cohort and (Bio)SPAR cohort had a mean age of 43.2 ± 12.7 years and 41.9 ± 12.8 years, respectively. Mean age of the early axSpA patients from the (Bio)SPAR cohort was 36.4 ± 11.7 years and was significantly lower (*p=0.0007*). In the UH cohort, 57% of axSpA patients were male as compared to 66% of all axSpA patients (*p=0.1571*) and 61% of early axSpA patients (*p=0.6273*) from the (Bio)SPAR cohort. Fifty-nine % of axSpA patients in the UH cohort were HLA-B27 positive, compared to 86% of all axSpA patients (*p=<0.0001*) and 83% of early axSpA patients (*p=0.0026*) from the (Bio)SPAR cohort. The mean disease duration of axSpA patients from the UH cohort was 2.8 ± 1.4 years, whereas all axSpA patients and early axSpA patients from the (Bio)SPAR cohort had a disease duration of respectively 10.4 ± 10.9 years (*p=<0.0001*) and 1.4 ± 1.7 years (*p=<0.0001*).

Healthy controls (n=94) had a mean age of 44.6 ± 18.4 years and 52 (55%) were male. Persons with CLBP (n=75) had a mean age of 45.5 ± 9.2 years, a mean duration of low back pain complaints of 10.7 ± 9.3 years, 34 (45%) were male, 67 (89.3%) were workers, 2 (2.7%) were smokers, 38 (50.7%) underwent previous rehabilitation and 17 (22.7%) used medication. In addition, the average results (mean ± SD) of questionnaires for pain perception (Numeric Pain Rating Scale (NPRS)), low back pain disability (Modified Oswestry Index (MODI)),

physical disability (Physical Activity Scale for Individuals with Physical Disability (PASIPD)) and spinal pain disability (Million's Visual Analogue Scale (MVAS)) were respectively 5.6 ± 1.7, 9.9 ± 4.8, 15.4 ± 11.4 and 9.3 ± 0.8. RA patients (n=60) had a mean age of 51.6 ± 11.5 years, a mean disease duration of 3.1 ± 1.2 years, 30 (50%) were male and 57 (95%) received conventional disease modifying anti-rheumatic drugs (cDMARDs).

Identification of novel antibodies by serological antigen selection (SAS)

As a screening procedure, SAS was used to identify the antigenic targets of novel antibodies in early axSpA patients. To this end, a human axSpA cDNA phage display library was constructed from synovial hip tissues of 3 different axSpA patients, according to the previously described procedure [15]. Subsequently, phage particles expressing randomly formed peptides and axSpA synovial antigens were used to screen for novel IgG antibodies present in the early axSpA SAS pool, using the previously described SAS procedure [16-19] with some minor modifications. To prevent the isolation of common antibodies, present in HC, counter selection was performed with the HC SAS pool. Peptides or proteins displayed on the surface of selected phage clones were identified by sequencing the fusion of M13 gene VI with the cDNA inserts [15]. The custom-made DNAnalyzer software, an Anaconda Python based multiprocessing program using Biopython [20], allowed automation of the comparison of nucleotide and amino acid (aa) sequences to human sequences with the BLAST tool of NCBI [21].

Detection of antibodies to 9 novel UH-axSpA peptides

Antibody reactivity against peptides displayed on phage particles was measured by phage ELISA in pooled or individual plasma samples as described previously [16, 18, 22], with some minor modifications. Here, half area 96- well Microlon high binding microplates

(Greiner, Belgium) were coated overnight at 4˚C with 3.5 µg/mL anti-M13 mouse monoclonal antibody (clone MM05T, Sino Biological, China) diluted in coating buffer (0.2 M sodium carbonate bicarbonate buffer, pH 9.6), and cross-adsorbed goat anti-human IgG-Fc conjugated with horseradish peroxidase (Bethyl, United States) diluted 1/10,000 in 5% (w/v) skimmed milk powder in phosphate-buffered saline (MPBS) was used as secondary antibody.

Antibody reactivity against each phage- displayed peptide is expressed by the ratio of the optical density (OD) signal of each phage- displayed peptide over the OD signal of the phage without peptide (OD(specific phage)/OD(empty phage)). For each peptide target, a cut-off for seropositivity was calculated as the mean of the antibody reactivity in the HC population plus 3*standard deviation (after single exclusion of outliers using the same formula).

For competition ELISA, plasma samples were pre-incubated with increasing concentrations (0- 30 µg/mL) of one of three synthetic peptides (>85% purity, GL Biochem, China), corresponding to the peptide sequence of UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8 (Table 2), before use in phage ELISA. For competition experiments, the peptide sequence of UH-axSp-IgG.4 was elongated with 6 N-terminal amino acids originating from the phage backbone (UH-axSpA-IgG.4A: ENSRPRIPELLLWKIQP). For the synthetic peptide sequence of UH-axSpA-IgG.8, only the part corresponding to the Double Homeobox protein 4 (DUX4) C-terminus was used (IPPGELEALEGATSLEAPLSEEEYRALLEEL). Increasing amounts of control peptide (WTKTPDGNFQLGGTEP) were used as a control.

Within each phage ELISA experiment, samples were tested in duplicate and experiments were performed independently at least twice. Average values of experimental repeats had a coefficient of variation (% CV) lower than 20%.

Immunohistochemical analysis of DUX4 expression in synovial tissue

Cryosections of synovial hip tissues of 3 axSpA patients and control synovial knee tissues of one RA patient and one healthy person with a sports injury were dried for 30 min at RT, fixated for 10 min in ice-cold acetone and dried for 30 min at RT. Synovial histology was revealed by the Masson trichrome staining. For fluorescent stainings, cryosections were washed three times with PBS and blocked for 30 min at RT with 100% protein block (Dako). Subsequently, mouse monoclonal anti-human DUX4 antibody (clone P4H2, Thermo Fisher Scientific), diluted 1/100 in PBS with 0.5% Triton X-100, was added to the sections. After overnight incubation in a humidified chamber at 4˚C, tissue sections were washed and incubated with goat polyclonal anti-mouse IgG secondary antibody conjugated with Alexa Fluor 555 (Life Technologies, Belgium) diluted 1/600 in PBS with 0.5% Triton X-100. Cell nuclei were counterstained in 300 nM 4',6-diamidino-2-phenylindol (DAPI; Thermo Fisher Scientific) for 10 min at RT and mounted with fluorescence mounting medium (Dako). As a negative control, no primary anti-DUX4 antibody was added.

Statistical analysis

All statistical analyses were performed using SAS JMP Pro version 13.2 and a p value of <0.05 was considered statistically significant. Presence of antibodies to particular UH-axSpA peptides or panels of peptides was compared between axSpA patients and controls by applying Fisher's exact test. Continuous clinical characteristics between antibody-positive and antibody-negative axSpA patients were compared using Student's t-tests, whereas categorical characteristics were compared by Fisher's exact tests.

The positive likelihood ratios (LR+) of antibodies against particular UH-axSpA peptides or panels of peptides were calculated based on their presence in axSpA patients and persons with CLBP. Applying the method described by Rudwaleit *et al.* [23], post-test probabilities could

be calculated based on the LR+ and the assumption that the prevalence of axSpA among patients with CLBP is 5%.

RESULTS

Screening for novel antibody targets in early axSpA patients

We constructed a human axSpA cDNA phage display library from synovial hip tissues of 3 axSpA patients with a large diversity of 1.88 x 10^6 recombinant bacterial clones, expressing (fragments of) known human proteins and randomly formed peptides. Using SAS to screen for axSpA immunoreactivity against the antigens in this axSpA cDNA phage display library resulted in the identification of novel IgG antibodies against 105 different peptides displayed on the phage surface.

Antibody reactivity against each of these 105 phage- displayed peptides was tested in 6 plasma pools of 10 early axSpA patients each and in 3 plasma pools of 10 HC each. As a result, for 9 of these 105 peptides, antibody reactivity was more frequently present in the axSpA plasma pools compared to the HC plasma pools (data not shown). These 9 peptides were called UH-axSpA-IgG.1 till UH-axSpA-IgG.9 (University Hasselt-axSpA-IgG isotype.target number).

Identity of UH-axSpA peptides targeted by novel axSpA antibodies

Nucleotide and amino acid (aa) sequences of the 9 selected UH-axSpA peptides were compared to human sequences using the custom DNAnalyzer program (Table 2). Peptide sequences expressed by UH-axSpA-IgG.1 to UH-axSpA-IgG.7 and UH-axSpA-IgG.9 had a length between 6 and 46 aa and resulted from the out-of-frame cDNA translation or translation of non-coding cDNA sequences and therefore showed only partial homology to different human proteins.

The DNA sequence of UH-axSpA-IgG.8 corresponded with an in-frame fusion to the 3'-coding region and 3'-untranslated region (UTR) of the human Double Homeobox protein 4 (DUX4) gene. Our isolated DUX4 variant showed 93% homology with the canonical DUX4 sequence (NM_001306068.2), resulting in the expression of a DUX4 C-terminal fragment with 26 to 29 aa homology (data not shown). Sequencing analysis of multiple additional synovial DUX4 transcripts showed that this gene was hypervariable in the region analysed. Sequencing of 34 different DUX4 transcripts, cloned from axSpA synovial mRNA, revealed 24 different variants with 1 to 9 single nucleotide polymorphisms over the 89 nucleotide sequence corresponding to the UH-axSpA-IgG.8 clone (results not shown). In addition, as amber stop codons (UAG) are translated to glutamine in the bacterial strain used to produce the phage clones, this sequence was followed by 221 aa resulting from the translation of the 3'-UTR sequence.

Expression of autoantibody target DUX4 in axSpA synovial tissue

To investigate the possible biological relevance of autoantibodies targeting UH-axSpA-IgG.8, representing the C-terminal part of DUX4, expression of DUX4 in synovial axSpA tissue was determined by immunohistochemistry. To our knowledge, presence of the DUX4 protein had not yet been described in synovial tissue. We showed clear DUX4 expression in synovial lining cells and fibroblasts in the underlying connective tissue in synovial hip tissue of each of three investigated axSpA patients (Figure 1A and results not shown). Within the synovial knee tissue of an RA patient, DUX4 expression was also present in the synovial lining layer (Figure 1B), whereas DUX4 expression was not detectable in the synovial lining of knee tissue from a healthy person without synovial inflammation (Figure 1C).

Presence of axSpA antibodies to 9 novel UH-axSpA peptides confirmed in two independent cohorts

Antibody reactivity against the 9 UH-axSpA peptides was further validated in individual plasma samples of early axSpA patients and controls from the UH cohort. Antibodies against individual UH-axSpA peptides were present in 3% (2/76) to 13% (10/76) of early axSpA patients, compared to 1% (1/94) to 6% (6/94) of HC, 0% (0/75) to 16% (12/75) of CLBP and 0% (0/60) to 12% (7/60) of RA patients (Table 3).

Presence of antibodies against these 9 UH-axSpA peptides was also confirmed in 174 axSpA patients from the independent (Bio)SPAR cohort, including 79 axSpA patients with an early disease course (Table 3). Within the (Bio)SPAR cohort, antibody reactivity against individual peptides was found in 2% (4/174) to 18% (32/174) of all axSpA patients, whereas antibody reactivity ranged from 1% (1/79) to 10% (8/79) in the early axSpA patients from this cohort.

Novel antibodies against 3 peptide targets are of added value for early axSpA diagnosis

We explored whether a combination of antibodies against particular peptides could be of added value in discriminating axSpA patients from persons with CLBP. From the 9 novel UH-axSpA peptides, we selected the three peptides with the highest LR+ in early axSpA patients as compared to persons with CLBP: UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8. Using competition ELISA with synthetic peptides corresponding to peptide target UH-axSpA-IgG.1, UH-axSpA-IgG.4A and UH-axSpA-IgG.8, it was confirmed that antibody reactivity was specifically directed towards the peptides expressed on the surface of these respective phage clones (Supplementary Figure 1). Although UH-axSpA-IgG.8 displayed a peptide of 251 aa, a synthetic peptide of only the first 31 aa, corresponding to the C-terminal part of DUX4, was sufficient for full competition.

Antibodies against at least one of these 3 UH-axSpA peptides were present in 14.2% (22/155) of early axSpA patients from the combined UH and (Bio)SPAR cohorts and only in 5% (4/75) of persons with CLBP (*p=0.0484*), resulting in a LR+ of 2.7 (Table 4). In addition, these antibodies were also present in 10.0% (6/60) of RA patients (*p=0.5025*) and in 8.4% (8/95) of HC (*p=0.2292*). Moreover, clinical and disease characteristics, as shown in Table 1, were compared between axSpA patients that tested positive for antibodies against this panel of 3 UH-axSpA peptides (UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8) and axSpA patients that were seronegative for this panel. We could not detect a significant difference in age, gender, HLA-B27 status, disease duration, treatment, Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), erythrocyte sedimentation rate (ESR), and CRP of early axSpA patients with or without antibody reactivity against this panel of 3 UH-axSpA peptides. Assuming a 5% prevalence of axSpA in persons with CLBP (pre-test probability), the combination of the presence of inflammatory back pain (LR+ 3.1) with a positive test result for the laboratory markers HLA-B27 (LR+ 9.0) and CRP (LR+ 2.5) [24], resulted in a disease (post-test) probability of 79% (Table 5). After addition of a positive test result for the presence of antibodies to the 3 UH-axSpA peptides (LR+ 2.7), post-test probability could be further increased to 91%. On the other hand, a combination of presence of inflammatory back pain (LR+ 3.1) with a positive test result for CRP (LR+ 2.5) provided a post-test probability of 27% in case of a negative test result for HLA-B27 (LR- 0.9) [24]. Addition of a positive test result for the presence of antibodies to the 3 UH-axSpA peptides (LR+ 2.7) increased the post-test probability to 49%.

DISCUSSION

In this study, we identified antibodies against 9 novel UH-axSpA peptides and confirmed their presence in axSpA patients and controls from two independent cohorts. Three of these 9

novel UH-axSpA peptides, UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8, showed promising potential in discriminating axSpA patients from persons with CLPB. In addition, DUX4, which shows expression in axSpA synovium, is proposed as a possible novel autoantigen in a subset of axSpA patients.

We used the antibody profiling technique SAS to identify the peptide targets of novel antibodies in early axSpA patients. First, a cDNA phage display library was constructed from synovial hip tissues of three axSpA patients. Initially, we also collected facet joint tissue of two axSpA patients, but the limited yield and quality of the isolated RNA from these tissues did not allow inclusion of this material for cDNA library construction.

From the 105 peptides identified by SAS, we selected 9 UH-axSpA peptides with the highest antibody reactivity in 6 axSpA plasma pools as compared to 3 HC plasma pools. Antibodies against each of these 9 selected UH-axSpA peptides were present in subsets of axSpA patients from the UH cohort, and the presence of these antibody reactivities could also be confirmed in axSpA patients from the independent (Bio)SPAR cohort. By combining antibody reactivity against the 3 peptides with the highest LR+, UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8, antibodies could be found in a subset of early axSpA patients (14.2%), with a corresponding specificity of 95% in CLBP. The resulting LR+ of 2.7 is a very similar value than the LR+ of CRP (2.5) [24], the only laboratory marker currently used in clinical practice to support axSpA diagnosis [3, 4]. At present, it is still challenging to distinguish between early axSpA patients and persons with CLBP at an early stage, as both groups suffer from low back pain and diagnostic criteria are lacking [5-7]. This might indicate the usefulness of our 3 antibody markers as an additional novel tool for the diagnosis of axSpA. On the other hand, antibodies against the 3 UH-axSpA peptides were not able to discriminate axSpA patients from RA patients or HC. However, HC normally do not show rheumatic and/or joint complaints and RA patients suffer from different clinical symptoms (e.g. symmetric synovitis

of multiple peripheral joints with joint tenderness, swelling and pain) [25, 26], distinguishing them from axSpA patients both at a primary care and at secondary rheumatology practices.

Presence of antibodies against the 3 UH-axSpA peptides was not correlated with a clinical phenotype within axSpA, but the role of this antibody panel in supporting the diagnosis lies in the combination of the different factors in the diagnostic process. Since antibody reactivity to this panel of 3 UH-axSpA peptides was not correlated with CRP or HLA-B27 status, these three laboratory tests can be used in parallel, each giving an independent, additional contribution to an increased post-test probability for axSpA diagnosis. The relevance of this is indicated by a post-test probability of 91% in early axSpA patients when adding the presence of antibodies against our 3 UH-axSpA peptides to the presence of CLBP, combined with a positive test result for the laboratory markers HLA-B27 and CRP (post-test probability of 79%) [24]. It should be noted that only a subset of all axSpA patients would have a positive test result for all these markers. Still, given the heterogeneous character of this disease, it is of interest to use a combination of laboratory and serological markers to provide a less ambiguous tool to improve the diagnosis of axSpA patients. Presence of antibodies against the 3 UH-axSpA peptides did not correlate with specific clinical characteristics, which is possibly due to the limited prevalence of these antibodies. Therefore, these antibodies do not seem to allow identification of subgroups of axSpA patients, at least not according to the clinical parameters used in this analysis. In the future, presence of antibody reactivity against our panel of 3 UH-axSpA peptides needs to be studied more extensively in larger cohorts of early axSpA patients and low back pain controls to determine their usefulness as clinical diagnostic biomarkers for axSpA.

The identification of antibodies to novel peptides and proteins is not only interesting from a clinical perspective, but might also be relevant to gain more insight in the biology and pathophysiology in axSpA, and the potential role of antibodies in axSpA. Eight of the 9 novel

UH-axSpA peptides displayed on phage were randomly formed peptides resulting from the out-of-frame expression of cDNA or expression of normally untranslated mRNA regions (e.g. 3'-UTR). These random peptides probably comprise epitopes that mimic *in vivo* antigen structures (mimotopes), which is one of the limitations of this study. At present, it is not known whether the antibodies against each of these 8 peptide targets are possible autoantibodies, formed against self-antigens, or antibodies formed as part of a normal immune reaction against for example a pathogen. These 8 UH-axSpA peptides showed partial aa homology to human proteins involved in several biological processes, such as protein biosynthesis (EIF4E2), lipid metabolism (PLCG2), regulation of MAPK cascade (GPR37L1), barrier function against particles and infectious agents at mucosal surfaces (MUC16), transcription regulation (NR1H3), protein transport (TTC26) and cell adhesion (FREM1). Still, none of these homologous proteins have been directly implicated in the pathogenesis of axSpA so far, and the identity of the *in vivo* antigens corresponding to the mimotopes will be subject of further study. Nevertheless, antibodies targeting such mimotope antigens can still result in interesting biomarkers with clinical relevance for the disease studied. In a similar approach, our research group previously identified antibodies against UH-RA.21, a mimotope peptide resulting from the translation of a 3'-UTR, as a novel candidate antibody biomarker for RA diagnosis [17, 18]. Antibody reactivity against UH-RA.21 was confirmed in RA patients from two independent cohorts and was associated with inflammation, joint erosion and higher tender and swollen joint counts [27]. These antibodies likely represent autoantibodies as they bind to RA synovial tissue slides [18], but the identity of the *in vivo* antigen targeted by these anti-UH-RA.21 antibodies remains subject of investigation.

In contrast, our competition experiment showed that anti-UH-axSpA-IgG.8 reactivity is directed against the C-terminus of the human DUX4 protein. The DUX4 C-terminal fragment of UH-axSpA-IgG.8 only showed 90% aa homology with the canonical DUX4 protein

sequence. This was in line with the large degree of variability we observed in the coding sequences of multiple DUX4 transcripts. This might be explained as the DUX4 gene is located within the D4Z4 macrosatellite repeat element [28], which is repeated 10 to 150 times and is subject to alternative splicing [29]. DUX4 expression has been described in human testis germline cells and is generally silenced in somatic tissues [30]. On the other hand, pathological expression has been shown in facioscapulohumeral muscular dystrophy [31] and in several solid tumors [32]. By amplifying DUX4 from synovial tissue RNA and by immunohistochemistry, we show for the first time that DUX4 is also highly expressed in the synovial lining layer of axSpA and RA synovium. On the other hand, DUX4 expression in the synovial lining layer could not be observed in synovial knee tissue from a healthy person with a sports injury, which has not been subject to chronic inflammation. Further confirmation of the inflammatory nature of this DUX4 expression in additional rheumatic patients and non-inflammatory controls, and the exact cell types involved, is needed in the future. Moreover, DUX4 acts as transcriptional activator and one of its main target genes encodes for the antimicrobial peptide beta-defensin 3 (BD3) [33], which regulates several immune related functions, such as co-stimulation [34] and chemotaxis [35, 36], and has been shown to activate matrix metalloproteinases [37]. However, at present, it is unclear whether DUX4 expression or autoantibodies against DUX4 might contribute to axSpA etiology.

In conclusion, in combination with other laboratory markers such as HLA-B27 and CRP, antibodies against our 3 UH-axSpA peptides, UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8, could provide a novel tool for the diagnosis of a subset of axSpA patients.

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TABLES

Table 1. Characteristics of axSpA patients from the UH and (Bio)SPAR cohorts used in this study.

		axSpA (Bio)SPAR cohort	
Clinical characteristics	**axSpA UH cohort**[a] (*n=76*)	**Total**[b] (*n=174*)	**Early**[c] (*n=79*)
Age (mean, SD)	43.2 (12.7)	41.9 (12.8)	36.4 (11.7)
Male (*n*, %)	43 (56.6)	115 (66.1)	48 (60.8)
HLA-B27 positive (*n*, %)	43 (58.9)	124 (86.1)	55 (83.3)
Disease duration in years[d] (mean , SD)	2.8 (1.4)	10.4 (10.9)	1.4 (1.7)
No medication use[e] (*n*, %)	11 (14.5)	13 (7.5)	5 (6.3)
NSAID use (*n*, %)	54 (71.1)	78 (70.3)	48 (77.4)
cDMARD use (*n*, %)	30 (39.5)	24 (21.6)	15 (24.2)
bDMARD use (*n*, %)	18 (23.7)	93 (53.4)	30 (38.0)
BASDAI (mean, SD)	4.7 (2.1)	4.4 (2.2)	3.9 (2.2)
BASFI (mean, SD)	4.3 (2.6)	3.6 (2.8)	2.8 (2.4)
ESR, mm/h (mean, SD)	11.5 (15.2)	19.1 (21.6)	19.3 (21.9)
CRP, mg/L (median, IQR)	2.5 (0.7- 7.8)	4.3 (1.3- 12.8)	5.3 (1.2- 15.6)
Extra-articular	15 (19.7)	34 (21.9)	17 (25.0)

manifestations[f]

(*n*, %)

[a] For axSpA patients in the UH cohort, missing values were below 10% except for BASDAI (26.3%), BASFI (27.6%), ESR (13.2%) and CRP (10.5%).

[b] Within the total (Bio)SPAR cohort, missing values were also below 10%, except for HLA-B27 status (17.2%), NSAID use (36.2%), cDMARD use (36.2%), BASDAI (38.5%), BASFI (39.1%) and extra-articular manifestations (10.9%).

[c] Missing values for axSpA patients in the early (Bio)SPAR cohort were below 10%, except for HLA-B27 (16.5%), NSAID use (21.5%), cDMARD use (21.5%), BASDAI (24.1%), BASFI (22.8%) and extra-articular manifestations (13.9%).

[d] Disease duration, time between diagnosis and blood sampling

[e] No medication use at the time of blood sampling

[f] Extra-articular manifestations, including uveitis, inflammatory bowel disease and psoriasis

HLA-B27, Human Leukocyte Antigen-B27; NSAID, Non-Steroidal Anti-Inflammatory Drug; cDMARD, conventional Disease-Modifying Anti-Rheumatic Drug; bDMARD, biological Disease-Modifying Anti-Rheumatic Drug; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; ESR, Erythrocyte Sedimentation Rate; CRP, C-Reactive Protein.

Table 2. Identity of 9 novel antigens targeted by antibody responses in early axSpA patients.

Antibody targets	**cDNA identity (NCBI Accession No.)**	**Fusion type[a], in frame[b]**	**Peptide sequence of cDNA insert[c]**	**Size (aa)[d]**	**Homology on amino acid level (UniProt Accession NO.)**
UH-axSpA-IgG.1	Tubulin gamma complex associated protein 2 (NM_001256617.1)	mRNA, coding, No	(IP)GPAEHL**Q**HQ*	11	8/10 (80%) Eukaryotic translation initiation factor 4E type 2, *EIF4E2* (O60573) 6/6 (100%) Adenylate cyclase type 8, *ADCY8* (P40145) 8/13 (62%) Cyclic AMP-responsive element-binding protein 3-like protein, *CREB3L4* (Q8TEY5)
UH-axSpA-IgG.2	RNA polymerase II associated protein 2 (NM_024813.3)	RNA, 3'-UTR	(WA)PTSKTKDR*	10	7/7 (100%) 1-phosphatidylinositol 4,5-biphosphate phosphodiesterase gamma-2, *PLCG2* (P16885) 7/10 (70%) WD repeat-containing and planar cell polarity effector protein fritz homolog, *WDPCP* (O95876) 6/6 (100%) G patch domain-containing protein 1, *GPATCH1* (Q9BRR8)
UH-axSpA-	Predicted: MAM	RNA	(WG)QAELMNKNGVG	46	7/9 (78%) D-amino acid

IgG.3	domain containing glycosylphosphatidylinositol anchor 1 (XR_926140.2)		**Q**KILHPLGLPNHLHRSFCPWLGLDFIRSFFWGR*		oxidase activator, *DAOA* (P59103) 10/14 (71%) Autism susceptibility gene 2 protein, *AUTS2* (Q8WXX7) 7/10 (70%) Leucine-rich repeat and IQ domain-containing protein 3, *LRRIQ3* (A6PVS8)
UH-axSpA-IgG.4	PREDICTED: Uncharacterized LOC105379031 (XR_953469.2)	ncRNA	(IP)ELLLWKIQP*	11	6/7 (86%) G-protein coupled receptor 37-like 1 precursor, *GPR37L1* (O60883) 6/9 (67%) 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, *COQ5* (Q5HYK3) 6/7 (86%) Rap guanine nucleotide exchange factor 1, *RAPGEF1* (Q13905)
UH-axSpA-IgG.5	MAGE family member D4 (NM_001272063.2)	mRNA, coding, No	(IP)ISTF*	6	5/5 (100%) Mucin-16, *MUC16* (Q8WXI7) 5/6 (83%) Activating transcription factor 7-interacting protein 1, *ATF7IP* (Q6VMQ6) 5/5 (100%) F-box only protein 47, *FBXO47* (Q5MNV8)
UH-axSpA-IgG.6	Malate dehydrogenase 2	mRNA, coding, No	(IR)QRCSPPQLQHLGPEQC*	18	9/13 (69%) Oxysterols receptor LXR-alpha, *NR1H3*

	(NM_005918.4)				(Q13133) 9/20 (45%) Transforming acidic coiled-coil-containing protein 2, *TACC2* (O95359) 7/10 (70%) Histone-lysine N-methyltransferase 2D, *KMT2D* (O14686)
UH-axSpA-IgG.7	Death associated protein 3 (NM_033657.2)	mRNA, coding, No	(WA)ESYFPHQ*	9	6/6 (100%) Intraflagellar transport protein 56, *TTC26* (A0AVF1) 8/15 (53%) Integrator complex subunit 10, *INTS10* (Q9NVR2) 5/5 (100%) TRAF3-interacting JNK-activating modulator, *TRAF3IP3* (Q9Y228)
UH-axSpA-IgG.8	Double Homeobox protein 4 (NM_001306068.2)	mRNA, coding, Yes	(IP)PGELEALEGATSLE APLSEEEYRALLEEL**Q**D ARLGRGRLRAGRWPLF RREHLAGYVGSCLPHA TSTGLTSLGFLPSRSRP GERLHTAETPHSGELPF FPGHPGASRLG**Q**RPDA LHLPLPCGGFRGHGLA RWSCPGFQFARCPGDL GSPDPAPPRTPLGSGW CKHTLALCPHLSGPRLS HSARARQAVALQVPVL PAFPQVQRPPRSLRVGE SPFQRSRGGVGKIPTCR GRLGHPRCRCGLAGLE GTAAAN*	252	26/29 (90%) Double Homeobox protein 4-like, *DUX4* (Q9UBX2) 21/47 (45%) 26S proteasome non-ATPase regulatory subunit 5, *PSMD5* (Q16401) 21/45 (47%) Phosphatidylinositol 4,5-bisphosphate 5-phosphatase A, *INPP5J* (Q15735)

UH-axSpA-IgG.9	28S ribosomal (NR_146154.1)	Ribosomal RNA	(LG)ARTKAAVAQ*	11	7/9 (78%) FRAS1-related extracellular matrix protein 1, *FREM1* (Q5H8C1) 6/8 (75%) Transmembrane and coiled-coil domains protein 1, *TMCC1* (O94876) 6/7 (86%) RIMS-binding protein 2, *RIMBP2* (O15034)

[a] Origin of the cDNA insert of the phage- displayed target

[b] In-frame fusion of the cDNA coding region with the M13 gene VI: Yes/No. Translation of in-frame fusion results in expression of (part of) a human protein, whereas out-of-frame fusion results in a fusion construct with a random peptide sequence.

[c] Peptide sequence of the translated cDNA insert, with the first two aa between parenthesis representing the transition between the M13 phagemid vector and the cDNA insert.

[d] Size of translated cDNA insert in aa

Q Amber stop codon, which is translated into glutamine by the bacterial strain

* stop codon

UTR, untranslated region; mRNA, messenger RNA; ncRNA, non-coding RNA.

Table 3. Presence of antibodies against individual peptide targets UH-axSpA-IgG.1-9 in axSpA patients and controls.

	UH cohort	(**Bio)SPAR cohort**	**Controls**

Antibody targets	**axSpA** (*n=76*)	**Total axSpA** (*n=174*)	**Early axSpA** (*n=79*)	**HC** (*n=94*)	**CLBP** (*n=75*)	**RA** (*n=60*)
UH-axSpA-IgG.1	2/76 (3%)	6/174 (3%)	3/79 (4%)	1/94 (1%)	1/75 (1%)	0/60 (0%)
UH-axSpA-IgG.2	3/76 (4%)	4/174 (2%)	1/79 (1%)	2/94 (2%)	2/75 (3%)	1/60 (2%)
UH-axSpA-IgG.3	5/76 (7%)	8/174 (5%)	5/79 (6%)	2/94 (2%)	3/75 (4%)	1/60 (2%)
UH-axSpA-IgG.4	6/76 (8%)	5/174 (3%)	3/79 (4%)	4/94 (4%)	0/75 (0%)	2/60 (3%)
UH-axSpA-IgG.5	10/76 (13%)	32/174 (18%)	8/79 (10%)	6/94 (6%)	12/75 (16%)	6/60 (10%)
UH-axSpA-IgG.6	6/76 (8%)	8/174 (5%)	4/79 (5%)	4/94 (4%)	3/75 (4%)	3/60 (5%)
UH-axSpA-IgG.7	2/76 (3%)	5/174 (3%)	1/79 (1%)	3/94 (3%)	4/75 (5%)	4/60 (7%)
UH-axSpA-IgG.8	4/76 (5%)	14/174 (8%)	6/79 (8%)	3/94 (3%)	3/75 (4%)	4/60 (7%)
UH-axSpA-IgG.9	7/76 (9%)	12/174 (7%)	4/79 (5%)	5/94 (5%)	5/75 (7%)	7/60 (12%)

Table 4. Presence of antibodies against UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8 peptides and our panel of 3 peptides in axSpA patients compared to persons with CLBP.

Antibody targets	**CLBP (*n=75*)**	**All axSpA (*n=250*)**			**Early axSpA (*n=155*)**		
	Spec (%)	**Sens (%)**	**p value**	**LR+**	**Sens (%)**	**p value**	**LR+**
UH-axSpA-IgG.1	1/75 (99%)	8/250 (3%)	0.6903	3.0	5/155 (3%)	0.6666	3.0
UH-axSpA-IgG.4	0/75 (100%)	11/250 (4%)	0.0744	-	9/155 (6%)	0.0328	-
UH-axSpA-IgG.8	3/75 (96%)	18/250 (7%)	0.4278	1.75	10/155 (6%)	0.5546	1.5
Panel of 3 peptides	**4/75 (95%)**	**34/250 (13.6%)**	**0.0637**	**2.6**	**22/155 (14.2%)**	**0.0484**	**2.7**

LR+, Positive Likelihood Ratio; Spec, Specificity; Sens, Sensitivity.

Table 4. Added value of our antibody panel of 3 antibodies to axSpA post-test probability.

Test combination	**axSpA post-test probability**
HLA-B27 positive (LR+9.0)	
Inflammatory back pain (LR+3.1)	79% [24]
CRP (LR+2.5)	

HLA-B27 positive (LR+9.0)	
Inflammatory back pain (LR+3.1)	91%
CRP (LR+2.5)	
Panel of 3 UH-axSpA autoantibodies (LR+2.7)	
HLA-B27 negative (LR-0.9)	
Inflammatory back pain (LR+3.1)	27% [24]
CRP (LR+2.5	
HLA-B27 negative (LR-0.9)	
Inflammatory back pain (LR+3.1)	49%
CRP (LR+2.5)	
Panel of 3 UH-axSpA autoantibodies (LR+2.7)	

CRP, C-Reactive Protein; HLA-B27, Human Leukocyte Antigen B27; LR+, Positive Likelihood Ratio; LR-, Negative Likelihood Ratio; UH, Hasselt University.

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plasma/serum samples and clinical characteristics of axSpA patients and controls and Igna Rutten (Hasselt University - Faculty of Medicine and Life Sciences, Biomedical Research Institute and Transnationale Universiteit Limburg, Hasselt, Limburg, Belgium) for excellent technical support.

FIGURE LEGENDS

Figure 1. Presence of Double Homeobox protein 4 (DUX4) staining in axSpA and RA synovial tissues. (A, B) Staining for DUX4 showed presence of DUX4 in cells of the synovial lining layer (*arrows*) and cells with a fibroblast-like morphology in the connective tissue underlying the synovial lining layer (*arrowheads*) in axSpA hip and RA knee synovial tissue respectively (20x). (C) This DUX4 staining could not be found in knee synovial tissue of a person with a sports injury (20x). (D, E, F) Negative control staining without addition of primary DUX4 antibody to synovial tissues of an axSpA patient, an RA patient and a healthy person with a sports injury respectively (20x). (G, H, I) Masson Trichrome staining revealed the histology of synovial tissues of an axSpA patient, an RA patient and a healthy person with a sports injury respectively (20x).

Supplementary Figure 1. Successful competition between synthetic peptides corresponding to peptide target UH-axSpA-IgG.1, UH-axSpA-IgG.4A and UH-axSpA-IgG.8. In a phage ELISA, an antibody-positive (P) and antibody-negative (N) plasma sample were pre-incubated in solution with increasing amounts of synthetic peptide, corresponding to the respective cDNA product of UH-axSpA-IgG.1, UH-axSpA-IgG.4 and UH-axSpA-IgG.8 displayed on the surface of each phage clone. For UH-axSpA-IgG.4, the peptide sequence

was elongated with 6 N-terminal amino acids originating from the phage backbone (UH-axSpA-IgG.4A) and the peptide sequence of UH-axSpA-IgG.8 only consisted of the sequence part corresponding to the DUX4 C-terminus. As a negative control, both samples were pre-incubated with increasing amounts of non-relevant control peptide. Within antibody-positive (P) plasma samples, effective competition was detected for all three synthetic peptides tested (specific peptide), whereas no competition was seen after pre-incubation with the control peptide and for the antibody-negative (N) plasma samples.