Detection of Multiple Autoantibodies in Patients with Ankylosing Spondylitis Using Nucleic Acid Programmable Protein Arrays.

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Abbreviations: AS: Ankylosing Spondylitis, RA: Rheumatoid Arthritis, NAPPA: Nucleic Acid Programmable Protein Arrays, HLA: Human Leukocyte Antigen, RF: Rheumatoid Factor, GST: Glutathione-S-Transferase, TNF: Tumor Necrosis Factor, CD: Cluster of Differentiation, IL: Interleukin, Th: T helper, IVTT: in vitro Transcription-Translation, EBNA: Epstein Barr virus Nuclear Antigen.
Summary

Ankylosing Spondylitis (AS) is a common, inflammatory rheumatic disease, which primarily affects the axial skeleton and is associated with sacroiliitis, uveitis and enthesitis. Unlike other autoimmune rheumatic diseases, such as rheumatoid arthritis or systemic lupus erythematosus, autoantibodies have not yet been reported to be a feature of AS. We therefore wished to determine if plasma from patients with AS contained autoantibodies and if so, characterize and quantify this response in comparison to patients with Rheumatoid Arthritis (RA) and healthy controls. Two high-density nucleic acid programmable protein arrays expressing a total of 3498 proteins were screened with plasma from 25 patients with AS, 17 with RA and 25 healthy controls. Autoantigens identified were subjected to Ingenuity Pathway Analysis in order to determine patterns of signalling cascades or tissue origin. 44% of patients with Ankylosing Spondylitis demonstrated a broad autoantibody response, as compared to 33% of patients with RA and only 8% of healthy controls. Individuals with AS demonstrated autoantibody responses to shared autoantigens, and 60% of autoantigens identified in the AS cohort were restricted to that group. The AS patients’ autoantibody responses were targeted towards connective, skeletal and muscular tissue, unlike those of RA patients or healthy controls. Thus, patients with AS show evidence of systemic humoral autoimmunity and multispecific autoantibody production. Nucleic Acid Programmable Protein Arrays constitute a powerful tool to study autoimmune diseases.
Introduction

Ankylosing Spondylitis (AS) is a chronic, debilitating, rheumatic disease with a predilection for the axial skeleton and large joints. It affects in excess of 0.1% of the population and can be associated with uveitis, apical pulmonary fibrosis and cardiac disease [1, 2]. AS is difficult to diagnose and patients can suffer symptoms for years before receiving appropriate treatment [2]. The aetiology is unknown, but is thought to be immune mediated. The extremely strong association with the Class I Human Leukocyte Antigen allotype HLA-B27 has led to hypotheses involving CD8 T cell-mediated immunity [3]. More recently additional genetic associations, including IL23R, have suggested a role for Th17 T cells [4].

Autoantibodies are a common characteristic of many rheumatic autoimmune diseases [5]. Rheumatoid Factor (RF), an autoantibody against the Fc portion of IgG, occurs in more than 85% of patients with Rheumatoid Arthritis (RA). Although not specific to RA, RF is used routinely as a diagnostic test for RA and other autoimmune disorders [6]. More recently, autoantibodies to cyclic citrullinated peptides have proven more specific than rheumatoid factor in diagnosing RA and have been shown to have prognostic value [7].

Autoantibodies are not commonly considered to be a feature of AS. However, anti-leukocyte [8], anti-neutrophil [9], and autoantibodies to some collagen proteins have been reported [10]. Increased levels of circulating plasma cells have also been reported in AS patients [11], as well as evidence of hypergammaglobulinemia [12]. Aside from these findings, no comprehensive investigation into the presence of autoantibodies in patients with AS has been performed to date.

Protein microarrays are commonly used as tools for detecting protein-protein interactions, such as the binding of autoantibodies to their cognate antigens. However,
technical issues involving the cloning and purification of thousands of proteins, protein folding and stability, and the shelf life of protein arrays have, until now, made this a challenging task. A novel technology, referred to as the Nucleic Acid Programmable Protein Array (NAPPA) has recently surmounted these issues [13]. NAPPA involves the in situ transcription-translation of thousands of glycosylated proteins in close spatial proximity. Biotinylated cDNAs containing GST-tagged query proteins are immobilized onto glass slides. Anti-GST antibodies spotted adjacent the cDNA are used as capture molecules. To synthesize the proteins, slides are covered in a continuous layer of reticulocyte lysate. The C-terminal GST tag ensures that only full-length proteins are captured. Therefore, this novel protein microarray technology provides an ideal platform for the characterization of autoantibody responses in autoimmune diseases.

In this discovery stage study we wished to determine whether patients with Ankylosing Spondylitis demonstrated autoantibody responses, using two different NAPPA arrays expressing a total of 3498 proteins. We show that AS patients demonstrate multispecific autoantibody responses to several autoantigens, predominantly targeted towards connective tissue and skeletal proteins.
Experimental Procedures

**Patient information.** This study included 25 patients with Ankylosing Spondylitis attending a dedicated AS clinic run by Dr. Paul Bowness at the Nuffield Orthopaedic Centre, Oxford, who met the modified New York criteria [14], and 17 individuals with Rheumatoid Arthritis attending Dr. Bowness’ rheumatology outpatients clinic, who met the American College of Rheumatology revised criteria [15]. Ethical permission was obtained (Oxfordshire REC 06/Q1606/139) and all subjects gave informed consent. AS patients were HLA-B*2705 positive by DNA typing. The AS patients’ mean age was 42 and the range from 28 to 69. 16 were male, 14 received Non-Steroidal Anti-Inflammatory drugs (NSAIDS), eight Disease-Modifying Anti-Rheumatic Drugs (DMARDS) and none anti-TNF agents. The AS patients’ mean and standard deviation scores on the Bath Ankylosing Spondylitis Disease Activity Index, Bath Ankylosing Spondylitis Functional Index, and Bath Ankylosing Spondylitis Metrology Index scales were 5.8 +/- 2.6, 5.4 +/- 2.8, and 5.2 +/- 1.8 respectively. The RA patients’ mean age was 48 and the range from 18 to 68. Eleven were female, six received NSAIDs, eleven received DMARDs and none anti-TNF agents. The mean Disease Activity Score of the RA patients was 5.2 +/- 2.3. 25 healthy controls were studied (four HLA-B27+ individuals, and fourteen male) with a mean age of 40 and range of 28 to 64.

**Blood Samples and Plasma Isolation.** Venesection was performed using a 21-gauge needle and a 20 ml syringe. Heparinized blood samples were transferred into 50 ml falcon tubes. To isolate the plasma, the samples were centrifuged at 500 g for 10 minutes at room temperature. Isolated plasma was aliquoted in 1 ml fractions into 1.5 ml microcentrifuge tubes, and stored at –80°C for one to two years. All samples
underwent one freeze thaw cycle prior to analysis. All samples were collected contemporaneously.

**Preparation of NAPPA slides and DNA preparation.** Glass slides were treated with 2% aminosilane in acetone, washed with acetone and then water, and dried using forced air. Slides were then stored in a dry container with silica packs until ready for printing. Bacteria harbouring the expression plasmids were cultured in 1.5 ml of Terrific Broth containing 10% potassium phosphate and 100 µg/ml ampicillin in 96-well plates for 24 hours, then pelleted by centrifugation at 3000 g for 30 minutes. DNA was prepared according to published protocols [16]. DNA concentrations were measured by spectrophotometry at 260 nm and plates were deemed acceptable if 90% of the wells had a total of 15 µg or more.

**Preparation of DNA Samples and Array Printing.** DNA samples were precipitated by the addition of 0.8x volume of isopropanol and centrifugation at 4000 g for 30 minutes. They were then washed with 80% ethanol and allowed to air dry. Each well was resuspended in 20 µl of spotting buffer (50 µg/ml capture antibody, 3.6 mg/ml BSA, 2mM Bis Sulfsuccinimidyl Suberate) and mixed for 30 minutes. Sets of four 96 well plates were then transferred to one 384 well plate which was used for printing. Arrays were printed on the aminosilane treated glass slides using a Genetix Q Array2 printer. Standard conditions of 60% humidity were applied.

**NAPPA Protein Expression.** Printed slides were blocked for 1 hour at room temperature on a rocking platform in SuperBlock (Thermo Scientific Pierce) using 30 ml for four slides to wash away any unbound NAPPA reagents (plasmid, BSA, or
capture antibody). The slides were then rinsed with water and dried with filtered compressed air. 100 µL of rabbit reticulocyte lysate in vitro transcription-translation mix (IVTT) (Promega, Wisconsin) was prepared per slide (4 µL TNT buffer, 2 µL T7 polymerase, 1 µL of –Met, 1 µL of –Leu, 2 µL of RNaseOUT and 90 µL of DEPC water) and Hybriwell gaskets (Grace Biolabs, Oregon) were applied. IVTT mix was pipetted onto the array through the hole in the gasket. Port seals were applied to both ports to avoid evaporation. The arrays were incubated for 1.5 hours at 30°C then 30 minutes at 15°C for protein expression and binding the immobilized capture antibody. The HybriWells were removed and the arrays were washed with milk three times for 3 minutes on a rocking platform. The protein arrays were then blocked with milk at room temperature for 1 hour. Between-plate replicate protein spot concentrations had a correlation coefficient of 0.96 under these conditions [16].

**Antibody capture and visualization.** Plasma samples were diluted between 1/170 and 1/2000 in 2 ml of 5% milk in PBS to achieve an equal amount of non-specific background binding. To avoid any biases from irregularities in the spotting procedure only arrays from a single batch were used. Furthermore, the 67 serum samples from all three groups were coded, mixed and randomized to the 67 arrays. The samples were pipetted onto the arrays, assembled into gaskets (Corning) and incubated overnight while rotating at 4°C. The arrays were then disassembled and washed three times in milk for 5 minutes on a rocking platform. Secondary antibody (HRP-conjugated anti-human IgG) was applied to the slide under a cover slip and incubated for 1 hour at room temperature. The slides were washed in PBS three times for 5 minutes, once with water, and dried. Tyramide signal amplification solution covering the arrays was applied under a cover slip and incubated for 10 minutes at room
Arrays were then scanned in a micro array scanner, using settings for Cy3.

**NAPPA Data analysis.**

For each protein target query on the NAPPA arrays, the mean and standard deviation (SD) of the signal intensity of the 25 healthy controls were calculated. Z-scores were then calculated for every query protein spot in every individual in the three groups. 

$$Z\text{-score} = \frac{\text{Signal} - \text{Mean}}{\text{Standard Deviation}}.$$  

A positive “hit” was attributed if the Z-score was greater than three standard deviations above the healthy control mean intensity.

**NAPPA validation.** 50 µl of pooled plasma from four IL-6 autoantibody positive AS patients and four IL-6 autoantibody negative AS patients or eight healthy donors was diluted in 1 ml of PBS. 20 µl of protein A sepharose beads (Invitrogen) was added and the samples were mixed at room temperature. After one hour the beads were washed with 1 ml of each PBS, TBS-T, TBS-T + 0.5 M NaCl, TBS-T + 0.5 % Triton X100 and PBS. The beads were then blocked in 1 ml of 5 % milk in TBS-T for one hour at room temperature. 1, 10 or 100 ng of recombinant IL-6 (Miltenyi Biotec) was added to the samples. Controls were BSA (Sigma) coated and empty beads. The samples were mixed and incubated overnight at 4°C. After washing 3 times in 1 ml of TBS-T the bound proteins were eluted with Laemmli Buffer. 10% of the eluate was separated by a 16 % Tris-Glycin SDS-Page followed by immunoblotting. IL-6 was detected using a polyclonal antibody (R&D Systems). Densiometric analysis of the IL-6 signal was performed using ImageJ.
Results

Screening of AS and control plasma samples using two dedicated NAPPA arrays.

Prior to the NAPPA screening plasma samples were coded, blinded and randomized. Each sample was screened on a mini-array, consisting of 100 proteins, to measure background signal. All plasma samples were diluted to at least 1/170 to normalize for background intensity. Figure 1 shows the workflow of this study. After the proteins were synthesized in situ using a transcription-translation coupled rabbit reticulocyte lysate system, the slides were washed and blocked to minimize non-specific interactions between plasma proteins and those present on the array. The slides were incubated with plasma overnight at four degrees to permit binding of autoantibodies to their target antigens, and were then incubated with a secondary HRP-conjugated anti-human IgG antibody. Visualization was performed using a Tyramide-Cyanine 3 conjugated amplification system, and scanned using a slide fluorescence scanner. Signals for each spot were averaged over the cohort and a statistical distribution of the intensities of those signals among the controls was calculated. From these distributions means and standard deviations were calculated for each spot and individual. On each array positive controls included the Epstein-Barr virus EBNA protein and human IgG protein (to which the HRP-anti-human secondary antibody would bind). Negative controls included the parental expression vector containing the GST-tag but lacking a cDNA, as well as spots that carry the spotting mix but lacked any DNA.

Figure 2 shows the plasma screening result from one AS patient (AS4) and one healthy control (HC8) on NAPPA array GST #1. Seven autoantigens were detected in patient AS4 and none in control HC8. This array contained 1749 target proteins and was chosen because it expressed 279 proteins associated with
immunological disease, 297 proteins associated with connective tissue disorders, 273 proteins associated with inflammatory disease and 204 proteins involved in skeletal and muscular disorders. NAPPA array GST#2 also expressed 1749 target proteins, 220 involved in inflammatory disease and 196 involved in immunological disease, but expressed fewer proteins involved in connective tissue and skeletal and muscular disorders, 109 and 143 respectively.

44% of AS patients demonstrate multi-specific autoantibody responses. Each one of the 67 plasma samples (25 AS, 17 RA inflammatory controls and 25 healthy controls) was screened against two NAPPA arrays comprising a total of 3498 human proteins. These proteins are listed as supplementary data (table S1). The number of autoantigens detected in each individual plasma sample from the screening of NAPPA arrays GST#1 and GST#2 is listed in Tables 1 and 2 respectively. The AS patients’ plasma samples reacted consistently with more autoantigens than the RA or healthy controls. Thus, 28% (7/25) of AS patients, compared to 18% (3/17) of RA patients and 4% (1/25) of healthy controls bound at least 50 proteins from the screening of array GST#1. Similarly, from the screening of the array GST#2, 20% (5/25) of AS patients, 19% (3/16) of RA patients and 4% (1/25) of healthy controls demonstrated a multi-specific autoantibody response of 50 proteins. Combining arrays, 44% (11/25) of AS patients, 36% (6/17) of RA patients and 8% (2/25) of healthy controls demonstrated a response to more than 50 autoantibodies. From the screening of NAPPA array GST#1, 92% of healthy control individuals expressed fewer than five autoantibodies, compared with 48% of AS patients and 53% of RA patients. From the screening of NAPPA array GST#2, 70% of healthy control individuals expressed
fewer than five autoantibodies, compared with 36% of AS patients and 19% of RA patients.

**NAPPA method validation.** Although the NAPPA method is a highly reproducible and robust system for detecting protein-protein interactions [16], we wished to validate one of the NAPPA results via an independent experimental method. Eight AS patient plasma samples were pooled, in half of which IL-6 had been identified as a putative autoantigen, as were eight plasma samples from healthy control individuals. From these two pools IgG molecules were immunoprecipitated using protein A beads. These IgG immunoprecipitates were then tested in a dose-dependent manner for recovery efficiency of recombinant IL-6. After blotting 10% of the immunoprecipitated material for the presence of IL-6, densitometry analysis showed that IgG from the AS plasma pool recovered more than 60% of recombinant IL-6, compared with just over 5% recovery from the healthy control plasma pool (see figure 3).

**Multiple AS patients show autoantibody responses to shared autoantigens.** We next asked whether any of the autoantibodies were present in multiple plasma samples within the AS patient group. From the screening of NAPPA array GST#1, 193 autoantigens were shared by three AS patients, 82 autoantigens were shared by four AS patients, 30 autoantigens were shared by five AS patients and three autoantigens were shared by six AS patients (figure 4A). Data from array GST#2 was similarly analysed. Figure 4B shows that 130 autoantigens were detected in at least two AS patients, 18 autoantigens were detected in three AS patients and two autoantigens were detected in four AS patients. Fewer autoantibodies were present in multiple
plasma samples in the RA patients and no autoantibodies were found in multiple healthy control individuals from the screening of either the GST#1 or GST#2 arrays.

**60% of autoantibodies detected are specific to the AS cohort.** We then asked if the detected autoantibodies were largely common between AS and RA patients, or specific to the AS group. From the screening of array GST#1, 482 (62%) were specific to the AS patients (Figure 5A). The AS and RA cohort shared 256 common autoantigens. Results from the screening of array GST #2 (Figure 5B) were comparable, with 436 (58%) of these autoantibodies restricted to the AS cohort and 281 shared by the AS and RA groups.

**Autoantibodies from AS patients show a bias towards antigens involved in skeletal and connective tissue disorders.** We next asked if autoantigens detected in the AS cohort showed a bias towards any particular biological pathway. For this purpose we used Ingenuity Pathway Analysis® (IPA), which is based on a comprehensive collection of literature data on protein networks. Figure 6A shows that, when compared to the pathways assigned to the 1749 target proteins expressed by the array itself, the autoantigens identified in the AS patients demonstrated a distinct bias towards the pathway involving connective tissue development and function. The GST#1 array expressed 95 proteins involved in connective tissue development and function. Of these, 65 proteins (68%) were detected as autoantigens in the AS cohort. By contrast, only 0-37% of pathway proteins were recognized in the other 26 pathways assigned to the array. This was expressed by IPA as a more significant p-value for connective tissue development and function in the AS autoantigen analysis (p-value = 4.09 x 10^{-11}) compared to the analysis of the entire
GST#1 array (p-value = 1.54 x 10^{-10}) after correction for multiple comparisons. The same analysis, conducted on results from the independent screening of NAPPA array GST #2, was consistent with the analysis from NAPPA array GST #1, and showed that the autoantigens detected in the AS cohort were specifically biased towards connective tissue disorder and skeletal and muscular disorder pathways (see figure 6B). We next explored whether autoantigens from these significant pathways occurred in multiple AS patients and were restricted to the AS cohort. 83 such proteins were identified. Table 3 lists a subset of these autoantigens, specifically those involved in extra-cellular matrix and bone remodelling.
Discussion

We have used a novel type of protein array screening tool to characterize the autoantibody response in patients with Ankylosing Spondylitis. In total, 44% of AS patients demonstrated a broad autoantibody response, with over 750 reactivities seen at plasma dilutions of greater than 1/170, which is considered clinically significant for autoimmune disease [17]. AS patients demonstrated autoantibody responses to several shared autoantigens, and 60% of the autoantibodies in AS patients appeared specific to that group, in that they were not found in RA or healthy controls. Further evidence of biological relevance is provided by the fact that only autoantibodies from AS patients showed a bias towards autoantigens involved in skeletal and connective tissue. Our studies indicate that NAPPA is a powerful new technique to screen for autoantibodies in human autoimmune diseases. The use of NAPPA arrays has the advantage that large numbers of proteins can be screened and that these proteins are translated and transcribed in a eukaryotic cell extract, promoting proper folding and glycosylation. However, a disadvantage is that antibodies to proteins with post-translational modifications would not be detected. Similarly, epitopes whose conformation may be membrane-dependent might not be detected.

Autoantibodies are associated with many systemic autoimmune diseases and can be highly specific (e.g. myasthenia gravis) [18] or broadly recognize multiple specificities (e.g. systemic lupus erythematosus) [19]. Our data show for the first time that AS patients’ plasma contains multiple autoantibodies recognizing a variety of antigens, with a bias towards proteins expressed in connective tissue.
We propose two possible interrelated mechanisms for this. Firstly production of IL-17 by T cells in AS [20] may directly stimulate B cell maturation and Ig production, as shown for systemic lupus erythematosus by Doreau and colleagues [21]. Secondly, the presence of professional antigen presenting cells together with T and B cells within inflamed areas of connective tissue, as demonstrated histopathologically in AS [22], may lead to autoimmunity in the presence of appropriate cytokine stimulation. Interestingly, we identified and validated Interleukin-6, a cytokine implicated in both AS and the Th17 response, as a target autoantigen. Accumulating evidence from immune mediated diseases supports the hypothesis that the tissue damage caused by immune responses can result in “epitope spreading” following priming of self-reactive lymphocytes. Here presentation of pathogenic epitopes in draining lymph nodes leads to the migration of activated lymphocytes to the site of inflammation, recruiting more phagocytes, and contributing to further tissue destruction. The debris from this destruction can result in extracellular matrix and inflammatory self-proteins being proteolytically digested and their resultant peptides (inappropriately) presented by professional antigen presenting cells. This may lead to the activation of autoreactive T and B lymphocytes, perpetuating the cycle of inflammation and tissue destruction [23].

Autoantibodies to extracellular matrix (ECM) components such as collagen I, II, III, IV and V have previously been reported in AS patients [24, 25], and elevated levels of IgA antibodies to keratin proteins have been detected in patients with spondyloarthropathy [26]. Also, higher levels of IgA antibodies have been detected in immune complexes precipitated from AS patient plasma [27]. We were not able to look for autoantibodies to collagen proteins I-IV in the screening of the NAPPA GST#1 and GST#2 arrays, as they were not expressed (Table S1). Unfortunately, the
design of this study was not appropriate for the identification of known RA antigens for the following reasons: First, we were not able to identify Rheumatoid factor (an autoantibody against the Fc portion of IgG) in the RA cohort, as IgG proteins were included as positive controls for the function of the secondary antibody in the NAPPA system. Secondly, antibodies to citrullinated proteins and peptides (ACPA) are enzymatically post-translationally modified \textit{in vivo} during inflammation, and therefore not detected in the NAPPA system. However, we did identify several extracellular matrix proteins as autoantigens in multiple AS patients. These included connective tissue growth factor [28], glypican 3 [29], glypican 4 [30], matrix Gla protein [31], and SMOC1, a protein involved in extracellular matrix assembly [32]. These proteins were all specific to the AS cohort (see Table 3). Furthermore, in addition to proteins involved in extracellular matrix remodelling, proteins involved in ossification and bone remodelling were also identified as autoantigens in multiple AS patients, and were restricted to the AS cohort. For example, chondromodulin is a bone remodelling factor [33] that is thought to function by allowing cartilaginous tissue to be vascularized and replace by bone. The purinergic receptor P2RX7 is involved in ossification [34] and has been shown to regulate bone formation [35]. Similarly, the melanocortin 4 receptor has been shown to increase bone resorption [36]. Finally, the cartilage matrix protein osteoglycin induces ectopic bone formation [37] and the extracellular matrix glycoprotein osteonectin is involved in osteocyte differentiation and bone formation [38]. New bone formation is a characteristic feature of Ankylosing Spondylitis. It would be interesting to investigate whether a correlation between the presence of these autoantibodies and the ankylosis characteristic of late-stage AS existed.
It remains to be determined whether the autoantibodies observed in this study occur in early or late stage disease. The number of observed overlapping AS-specific autoantibodies is small, and may be a function the variability in disease symptoms presented by AS patients, as well as variability in disease activity, severity, duration and treatment within the cohort. Unfortunately, the cohort studied in this discovery stage experiment was too small to sub-classify the AS patients according to disease duration and activity, flares and remission, age, sex, or treatment and produce statistically significant results. If, as with diabetes, patients produced autoantibodies prior to the presentation of clinical symptoms, a smaller microarray specifically expressing hundreds of proteins involved in connective and skeletal tissue might prove to be a valuable diagnostic tool. Alternatively, if these autoantibodies occurred later as a result of disease activity, they may be of prognostic use for tailored treatment. For example, such AS patients may benefit from B-cell ablative therapy, which has recently been shown to improve clinical symptoms in patients with Rheumatoid Arthritis and dermatological autoimmune conditions [39]. Indeed, although we have not demonstrated a direct pathogenic role for the autoantibodies identified in AS, our study suggests a scientific rational for a therapeutic trial of B cell depletion in this disease.

In summary, the screening of two independent Nucleic Acid Programmable Protein Arrays with plasma from AS patients has shown consistent evidence of the presence of multiple autoantibodies compared to healthy controls and patients with RA. Both arrays demonstrated that AS patients produced autoantibodies to shared autoantigens, and that a number of these antigens were restricted to the AS cohort. Similarly, when subjected to Ingenuity Pathway Analysis, AS autoantigens from both arrays demonstrated a bias towards proteins expressed in connective and skeletal
tissue. This novel finding has implications for our understanding of the pathogenic processes underlying AS, and may in future aid diagnosis and treatment.
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Figure Legends

**Figure 1:** Schematic view of the workflow for NAPPA analysis used in this study. See text for details.

**Figure 2:** Representative screenings on NAPPA array GST #1 of one AS patient plasma sample (AS4) (A and B) compared to one healthy control plasma sample (HC8) (C and D). A) Screening of one AS plasma sample with seven autoantigens (ringed spots) annotated. MAPK13: mitogen activated protein kinase 13, APLP2: amyloid beta precursor-like protein 2, DSCR2: proteasome assembly chaperone 1, TRIM27: tripartite motif-containing 27, FCGRT: Fc fragment of IgG receptor transporter, HSPA2: heat-shock 70 kDa protein 2, SULT2A1: alcohol sulfotransferase B) Magnified section of A boxed in red. C) Screening of one HC plasma sample with no autoantigens detected. Ringed spots for comparative purposes only. D) Magnified section of B boxed in green. Colour code for array intensity ranges from least intense; blue < green < yellow < orange < red; most intense.

**Figure 3:** AS patient plasma can immunoprecipitate IL-6. 1, 10 and 100 ng of recombinant IL-6 was used as bait and immunoprecipitated with IgG molecules from 50 µl of pooled AS patient or control plasma samples. 10% of the immunoprecipitated material was separated by 16% SDS-PAGE and detected by anti-IL-6 immunoblotting.
**Figure 4:** *AS patients have autoantibodies to a number of common antigens.* Number of autoantibodies detected in 2-5 AS patients from the screening of GST#1 (A) and 2-4 AS patients from the screening of GST#2 (B).

**Figure 5:** *Most AS autoantibodies are not found in RA or healthy controls.* A) Venn diagram showing distribution of all autoantigens detected in the screening of NAPPA array GST#1 shows 482 (62%) of autoantigens detected were specific to AS patients. B) Screening of NAPPA array GST#2 shows 436 (58%) of autoantigens were restricted to the AS cohort.

**Figure 6:** *Ingenuity Pathway Analysis (IPA) comparison of proteins expressed by the NAPPA arrays and autoantigens detected in the AS cohort demonstrates a bias towards antigens involved in connective tissue and skeletal antigens.* A) IPA compared the p-values calculated to significant pathways assigned to all proteins expressed on the NAPPA array GST#1 to the p-values calculated to significant pathways assigned to all autoantigens detected in the AS cohort only. The p-value for the analysis of AS autoantigens in the connective tissue development and function pathway (p-value = 4.09 x 10^{-11}) was found to be more significant than that from the analysis of all proteins expressed on NAPPA array GST#1 (1.54 x10^{-10}). B) IPA compared the p-values calculated for significant pathways assigned to all proteins expressed on the NAPPA array GST#2 to the p-values calculated to the significant pathways assigned to all autoantigens detected in the AS cohort only. P-values obtained from the analysis of AS autoantigens in the connective tissue disorders (p = 5.7 x 10^{-4}) and skeletal and muscular disorders (p = 2.55 x 10^{-5}) pathways from the NAPPA GST#2 screen were lower than in the analysis of all proteins expressed on
NAPPA array GST#2 for those pathways, with the array p-value for connective tissue disorders of $6.82 \times 10^{-2}$ and skeletal and muscular disorders $1.22 \times 10^{-2}$. All p-values were calculated using the right tailed Fischer’s exact test and corrected for multiple comparison with the Benjamini-Hochberg method. * Denotes the pathway significance threshold where $p = 0.05$. 
### Tables

#### Table 1. Number of putative autoantigens detected per individual in the screening of NAPPA array GST#1.
For each target protein, the mean and standard deviation of the control cohort was calculated. Z scores \((\text{Signal} – \text{Mean})/(\text{Standard Deviation})\) were calculated for every query protein in every individual in the three groups. Proteins with Z-scores greater than three are assigned as positive hits.

| Healthy Control | # of Putative Autoantigens detected | AS Patient | # of Putative Autoantigens detected | RA Patient | # of Putative Autoantigens detected |
|-----------------|------------------------------------|------------|------------------------------------|------------|------------------------------------|
| HC1             | 0                                  | AS1        | 10                                 | RA1        | 1                                  |
| HC2             | 0                                  | AS2        | 3                                  | RA2        | 231                                |
| HC3             | 0                                  | AS3        | 3                                  | RA3        | 5                                  |
| HC4             | 0                                  | AS4        | 7                                  | RA4        | 12                                 |
| HC5             | 0                                  | AS5        | 136                                | RA5        | 120                                |
| HC6             | 1                                  | AS6        | 0                                  | RA6        | 39                                 |
| HC7             | 1                                  | AS7        | 246                                | RA7        | 3                                  |
| HC8             | 0                                  | AS8        | 2                                  | RA8        | 1                                  |
| HC9             | 7                                  | AS9        | 11                                 | RA9        | 0                                  |
| HC10            | 0                                  | AS10       | 219                                | RA10       | 0                                  |
| HC11            | 0                                  | AS11       | 152                                | RA11       | 11                                 |
| HC12            | 2                                  | AS12       | 2                                  | RA12       | 3                                  |
| HC13            | 1                                  | AS13       | 218                                | RA13       | 3                                  |
| HC14            | 0                                  | AS14       | 7                                  | RA14       | 130                                |
| HC15            | 0                                  | AS15       | 3                                  | RA15       | 0                                  |
| HC16            | 1                                  | AS16       | 0                                  | RA16       | 10                                 |
| HC17            | 1                                  | AS17       | 1                                  | RA17       | 2                                  |
| HC18            | 0                                  | AS18       | 158                                | RA18       |                                    |
| HC19            | 1                                  | AS19       | 26                                 | RA19       |                                    |
| HC20            | 0                                  | AS20       | 2                                  | RA20       |                                    |
| HC21            | 0                                  | AS21       | 6                                  | RA21       |                                    |
| HC22            | 0                                  | AS22       | 94                                 | RA22       |                                    |
| HC23            | 182                                | AS23       | 2                                  | RA23       |                                    |
| HC24            | 0                                  | AS24       | 0                                  | RA24       |                                    |
| HC25            | 1                                  | AS25       | 3                                  | RA25       |                                    |

#### Table 2. Number of putative autoantigens detected per individual in the screening of NAPPA array GST#2.
For each target protein, the mean and standard deviation of the control cohort was calculated. Z scores \((\text{Signal} – \text{Mean})/(\text{Standard Deviation})\) were calculated for every query protein in every individual in the three groups. Proteins with Z-scores greater than three are assigned as positive hits. ND denotes sample not done, due to experimental error.

| Healthy Control | # of Putative Autoantigens detected | AS Patient | # of Putative Autoantigens detected | RA Patient | # of Putative Autoantigens detected |
|-----------------|------------------------------------|------------|------------------------------------|------------|------------------------------------|
| HC1             | 1                                  | AS1        | 12                                 | RA1        | 202                                |
| HC2             | 0                                  | AS2        | 3                                  | RA2        | 32                                 |
| HC3             | 0                                  | AS3        | 2                                  | RA3        | 22                                 |
| HC4             | 1                                  | AS4        | 3                                  | RA4        | 1                                  |
| HC5             | 0                                  | AS5        | 19                                 | RA5        | 38                                 |
| HC6             | 11                                 | AS6        | 75                                 | RA6        | 44                                 |
| HC7             | 0                                  | AS7        | 11                                 | RA7        | 2                                  |
| HC8             | 22                                 | AS8        | 19                                 | RA8        | 97                                 |
| HC9             | 3                                  | AS9        | 59                                 | RA9        | 19                                 |
| HC10            | 2                                  | AS10       | 32                                 | RA10       | 28                                 |
| HC11            | 0                                  | AS11       | 11                                 | RA11       | 7                                  |
| HC12            | 23                                 | AS12       | 4                                  | RA12       | 7                                  |
| HC13            | 8                                  | AS13       | 34                                 | RA13       | 1                                  |
| HC14            | 2                                  | AS14       | 28                                 | RA14       | ND                                 |
| HC15            | ND                                 | AS15       | 2                                  | RA15       | 69                                 |
| HC16            | 0                                  | AS16       | 370                                | RA16       | 10                                 |
| HC17            | 9                                  | AS17       | 35                                 | RA17       | 32                                 |
| HC18            | 0                                  | AS18       | 144                                | RA18       |                                    |
| HC19            | 3                                  | AS19       | 1                                  | RA19       |                                    |
| HC20            | 0                                  | AS20       | 2                                  | RA20       |                                    |
| HC21            | 39                                 | AS21       | 15                                 | RA21       |                                    |
| HC22            | 101                                | AS22       | 10                                 | RA22       |                                    |
| HC23            | 0                                  | AS23       | 3                                  | RA23       |                                    |
| HC24            | 1                                  | AS24       | 1                                  | RA24       |                                    |
| HC25            | 2                                  | AS25       | 142                                | RA25       |                                    |
Table 3. Autoantigens involved in skeletal and connective tissue remodelling restricted to the AS cohort.

| Autoantigen                  | Number of patients detected in | Subcellular Location | Function*                                                                 | Reference |
|------------------------------|--------------------------------|----------------------|---------------------------------------------------------------------------|-----------|
| P2RX7                        | 4                              | Plasma membrane      | Ion channel, increases bone mineralization and ossification               | [31,32]   |
| Chondromodulin 1             | 4                              | Cartilage matrix     | Bone resorption & remodelling                                             | [30]      |
| Osteoglycin                  | 3                              | Extracellular Matrix | Induces ectopic bone formation                                             | [34]      |
| Melanocortin 4 receptor      | 3                              | Plasma membrane      | Increases bone resorption                                                 | [33]      |
| Osteonectin                  | 2                              | Extracellular Matrix | Bone formation and remodelling                                             | [35]      |
| Connective Tissue Growth Factor | 2                         | Cell surface, extracellular Matrix | Ossification, cartilage condensation                                       | [25]      |
| Glypican 3                   | 2                              | Extracellular Matrix | Mediates osteogenesis                                                     | [26]      |
| Glypican 4                   | 2                              | Extracellular Matrix | ECM structural protein                                                    | [27]      |
| Matrix Gla Protein           | 2                              | Extracellular Matrix | Regulation of ECM calcification                                           | [28]      |
| SMOC1                        | 2                              | Extracellular Matrix | Involved in ECM assembly                                                 | [29]      |

**Supplementary Table**

Table S1: 3498 proteins expressed by NAPPA arrays GST#1 and GST#2.
Spotting cDNA (GST-tagged) onto arrays

IVTT proteins captured by α-GST Abs on array

Incubation of arrays with plasma samples

Normalizing background signal by testing different plasma dilutions

Visualisation using Cy3-conjugated α-human IgG Ab

Pathway (IPA) analysis to detect signaling cascades and tissue origin
Figure 2

A B C

MAPK13 APLP2 DSCR2

AS Patient

AS Patient

CTRL

Control
Figure 3

| Bait IL-6 [ng] | 1  | 10  | 100 | 1   | 10  | 100 |
|----------------|----|-----|-----|-----|-----|-----|
| IL-6 [ng]     | 10 | 20  | 50  |     |     |     |

| IP (10% loading) |
|------------------|
| Recombinant IL-6 |
| HC               |
| AS               |
Figure 4

A

Autoantigens occurring in multiple AS patients from the screening of NAPPA array GST#1

B

Autoantigens occurring in multiple AS patients from the screening of NAPPA array GST#2
Figure 5
A
NAPPA Array GST #1
AS
226
30
28
99
HC
RA
205

B
NAPPA Array GST #2
AS
436
36
153
HC
RA
267
14
25
171
Figure 6

IPA Pathways Assigned to AS Putative Autoantigens from the screening of NAPPA Array GST#1

IPA Pathways Assigned to NAPPA Array GST#1

IPA Pathways Assigned to AS Putative Autoantigens from the screening of NAPPA Array GST#2

IPA Pathways Assigned to NAPPA Array GST#2