2015•2016
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Identification of new biomarkers for the diagnosis of Bechterew's disease

Promotor :
Prof. dr. Veerle SOMERS

Copromotor :
dr. Patrick VANDORMAEL

Marthe Quintens

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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List of abbreviations

2xTYAG	2xTY-ampicillin-glucose	**mNYC**	modified New York criteria
2xTYAK	2xTY-ampicillin-kanamycin	**MRI**	magnetic resonance imaging
100bp L	100 base pair ladder	**NaCl**	sodium chloride
Ab	antibody	**nr-axSpA**	non-radiographic axial spondyloarthritis
AS	Ankylosing Spondylitis	**nt**	nucleotide
ASAS	Assessment of Spondyloarthritis International Society	**OD**	optical density
axSpA	axial spondyloarthritis	**PBS**	phosphate buffered saline
bp	base pair	**PBS-M**	phosphate buffered saline-marvel
cfu	colony forming units	**PBS-T**	phosphate buffered saline-tween
CRP	C-reactive protein	**PCI**	phenol:chloroform:isoamyl alcohol
CV	Coefficient variance	**PEG**	polyethylene glycol
ds	double stranded	**pSpA**	peripheral spondyloarthritis
E. coli	*Escheria coli*	**RA**	rheumatoid arthritis
EDTA	ethylenediaminetetraacetic acid	**RIN**	RNA integrity number
ELISA	enzyme linked immunosorbent assay	**rRNA**	ribosomal RNA
ESR	erythrocyte sedimentation rate	**RT**	Room temperature
EtOH	ethanol	**SAS**	serological antigen selection
FU	fluorescence units	**SD**	standard deviation
H_2SO_4	sulfuric acid	**SI**	sacroilliac
HC	healthy controls	**smartL**	smart ladder
HLA	human leukocyte antigen	**SN**	supernatant
HRP	horseradish peroxidase	**SpA**	spondyloarthritis
IBD	inflammatory bowel disease	**TMB**	3,3',5,5' tetramethyl-benzidine hydrochloride
IBP	inflammatory back pain	**TNF**	tumor necrosis factor
IL	interleukin	**UTR**	untranslated region
MHC	major histocompatibility complex		

Abstract

Introduction: Ankylosing spondylitis (AS), or Bechterew's disease, is a chronic inflammatory rheumatic disorder primarily affecting the spine and sacroiliac joints. Since specific serological tests and pathognomonic clinical features are missing, the diagnosis of AS is often delayed for many years. Even though the humoral immune response is not a classic hallmark of the disease, emerging evidence suggests a possible role in the etiology of AS. Therefore, we aim to identify novel antibody (Ab) biomarkers for the diagnosis of AS.

Material & methods: The construction of a human AS cDNA phage display library was optimized. Pending on this optimization, the composition of an existing human rheumatoid arthritis (RA) cDNA phage display library was determined. This RA cDNA library has been used to screen for AS specific Ab reactivity using serological antigen selection (SAS) against antibodies in pooled plasma of early AS patients and against healthy controls (HCs), age and gender matched. Using a phage enzyme-linked immunosorbent assay (ELISA), resulting Ab targets were characterized for Ab reactivity in pooled and individual plasma samples of AS patients and HCs.

Results: The diversity of the human AS cDNA phage display library must be optimized prior to be used in further screening techniques. The human RA cDNA phage display library was used instead, as we proved that it is a cDNA library of good quality with a high diversity. Next we detected the presence of antibodies in AS patients, indicating the humoral immune response to be involved. Screening of the RA display library resulted in 67 different Ab targets. Using phage ELISA, screening of Ab reactivity against these Ab targets resulted in 21 targets with a heterogeneous reactivity in early AS patients and not in HCs.

Discussion & conclusions: Our results indicate the presence of Ab reactivity in AS patients, suggesting the involvement of the humoral immune response. The 21 antigen targets look very promising as candidate diagnostic biomarkers for AS, however their accuracy will be further validated in a bigger screening using more AS patients, other rheumatic patients and HCs. In conclusion, the 21 targets have the potential to be developed into novel biomarkers for the improved diagnosis of AS.

Samenvatting

Ankyloserende spondylitis (AS), oftewel de ziekte van Bechterew, is een chronische inflammatoire reumatische aandoening die vooral de ruggengraat en de sacro-iliacale gewrichten aantast. Omdat specifieke serologische testen en een pathognomisch kenmerk ontbreken voor AS, is de diagnose vaak met verschillende jaren vertraagd. Door deze vertraging kan onomkeerbare schade, zoals volledige verbening van de ruggengraat, optreden, alsook een verminderde effectiviteit van de huidige behandeling. Dit beïnvloedt zowel de functionaliteit en de levenskwaliteit van de patiënten in negatieve zin. Hoewel de humorale immuunrespons niet kenmerkend is voor de ziekte, wijzen de resultaten van verschillende nieuwe onderzoeken op een mogelijke rol van B cellen en antilichamen in het ziekteproces van AS. Om die reden is de hypothese dat nieuwe antilichaam biomerkers, die de diagnose van AS versnellen, geïdentificeerd kunnen worden.

Tijdens dit project werden de verschillende stappen voor het creëren van een menselijke AS cDNA faag display bibliotheek geoptimaliseerd. In afwachting van de aanmaak van de AS cDNA faag display bibliotheek, werd een bestaande humane reumatoïde artritis (RA) cDNA faag display bibliotheek gekarakteriseerd en gebruikt voor het detecteren van AS specifieke antilichaam reactiviteit. Eerder werd antilichaam reactiviteit tegen bepaalde antigenen aangetoond door middel van serologische antigen selectie (SAS) in gepoold plasma van vroege AS patiënten en gezonde controles (GC) met overeenkomstige leeftijd en geslacht. Met behulp van een faag 'enzyme-linked immunosorbent assay' (ELISA), werden de resulterende antigenen gekarakteriseerd voor antilichaam reactiviteit in gepoold en in individuele plasmastalen van AS patiënten en GC.

Voordat de humane AS cDNA faag display gebruikt kan worden in verdere screeningstechnieken, moet deze verder geoptimaliseerd worden. De humane RA cDNA faag display bibliotheek werd dan gebruikt, die een goede kwaliteit en een hoge diversiteit bevat. Daarnaast hebben we de aanwezigheid van antilichamen in AS patiënten gedetecteerd, wat de mogelijke betrokkenheid van de humorale immuunrespons in AS suggereert. Screening van de RA display bibliotheek resulteerde in 67 verschillende antigenen. Met behulp van faag ELISA, werd antilichaam reactiviteit tegen deze antigenen getest, wat resulteerde in 21 antigenen met een heterogene reactiviteit in vroege AS patiënten en geen reactiviteit in GCs.

Onze resultaten tonen de aanwezigheid van antilichaam reactiviteit in AS patiënten aan, wat duidt op een mogelijke betrokkenheid van de humorale immuunrespons in AS. De 21 antigenen lijken veelbelovend te zijn als mogelijke diagnostische kandidaat biomerkers voor AS, hoewel hun diagnostische waarde verder gevalideerd moet worden in een grotere screening met meer AS patiënten, andere reumatische patiënten en GCs. In conclusie, de 21 antigenen hebben het potentieel om nieuwe antilichaam biomerkers te worden voor de verbeterde diagnose van AS.

1

1. Introduction

1.1. Ankylosing spondylitis

Spondyloarthritis (SpA) is a heterogeneous group of chronic inflammatory rheumatic diseases with overlapping clinical symptoms, in which the main disease characteristic is the involvement of the spine (1). SpA patients have a strong genetic link with the major histocompatibility complex (MHC) class I molecule human leukocyte antigen (HLA)-B27 (prevalence of HLA-B27 in SpA patients: 35–90%) (2-4). SpA discriminates itself from rheumatoid arthritis (RA) by the overlapping clinical symptoms between the different SpA subtypes and their genetically link (2).

Based on the predominance of presenting symptoms, SpA can be divided into a more axial (axSpA) or peripheral (pSpA) disease type. Peripheral SpA involves mainly the peripheral joints and includes reactive arthritis, psoriatic arthritis, arthritis with inflammatory bowel disease and undifferentiated SpA. On the other hand, axSpA is more characterized by inflammation occurring at the spine and sacroiliac (SI) joints. Based on radiological imaging, axSpA can be further divided in a radiographic and non-radiographic disease subtype (1). For this project, the focus will be on ankylosing spondylitis (AS), the radiographic form and prototype of SpA, which is more commonly known as Bechterew's disease (2).

The initial symptom of AS is a dull pain felt deep in the buttock and/or the lower back region and the presence of morning stiffness in the same region. This morning stiffness can last for a few hours and improves with activity. In a later stadium of the disease, these symptoms become more persistent and evoking pain, which evolves more bilateral resulting in chronic back pain (2). Next, sacroiliitis and inflammation at other locations in the axial skeleton emerge, resulting in the most predominant symptom of AS: inflammatory back pain (IBP) (3). In a more advanced stage of the disease, by combination of the chronic inflammation and new bone formation, the spinal vertebrae can even be painfully fused together to form the so called 'bamboo spine'. This spinal stiffness and resulting loss of spinal mobility, lead to the name of the disease in Greek terms: ankylose and spondylos, meaning 'stiff vertebrae' (3, 5). This ankylosis, irreversible stiffness of the joints, together with the sacroiliitis can become visible on conventional radiographs after some months to many years (2, 3).

Aside from these predominant symptoms, peripheral joints and extra-articular structures can also be affected in AS patients and can even emerge as first signs (2). Peripheral arthritis, inflammation of entheseal sites (bony sites of the tendons), eye inflammation like anterior uveitis, psoriasis, inflammatory bowel disease (IBD) and colitis are examples of these manifestations (3). In an Belgian study of Vander Cruyssen *et al.,* 42% of the studied AS population showed extra-articular manifestations, of which 51% was anterior uveitis, 20% psoriasis, 19% IBD and 10% a combination (6).

All the outcomes of AS can result in serious impairment of the spinal mobility and physical function, decreasing the functionality and quality of life of the patient (3, 7).

1.2. Epidemiology

Approximately, 1.5 million people suffer from AS within the European Union and the global prevalence of AS is believed to be between 0.1% and 1.4%, with most data coming from Europe, as the disease occurs more frequently within Europe and Asia (1-3, 7, 8). This prevalence has a close correlation with the prevalence of the class I MHC allele HLA-B27 in a given population. Still, this correlation also varies with ethnicity, as 90% of the Caucasian AS patients is positive for this risk allele and 50% of the African-American patients (9).

AS affects young people with an average age of onset of 28.3 years (2, 8). Eighty % of the patients develop the first symptoms at an age younger than 30 years and less than 5% of the patients do this at an age older than 45 years (10). However, a small percentage of patients starts developing symptoms during childhood or adolescence (5). This juvenile AS is mostly associated with worse functional disease outcomes (11, 12).

Another epidemiologic characteristic of AS is the disparity that exists between genders for AS with a mean gender ratio of 3.4:1 (male:female) (7). This gender difference manifest also in the disease progression and the following outcomes, as the radiological changes differ for men and women. Especially young HLA-B27 positive men progress more frequently to ankylosis of the spine as compared to women, however chronic back pain is the main symptom of AS for both genders (13, 14).

1.3. Pathogenesis

AS is a multifactorial disease developing from a complex interaction of genetic risk factors and environmental triggers (1). This multifactorial nature hinders the search for a main culprit in the pathogenesis of AS, despite numerous studies and hypotheses (1, 3).

1.3.1. Genetics

Resulting from twin studies, the risk to develop AS is largely genetically determined. Less than 50% of the overall genetic risk is caused by HLA-B27 (15, 16). Different underlying mechanisms are proposed for this association. First, the arthritogenic peptide hypothesis exist, where HLA-B27 presents antigens from microorganisms that trigger an autoimmune response. Next, it is suggested that the heavy chains of HLA-B27 form homodimers, which bind to regulatory immune receptors like natural killer receptors (the HLA-B27 heavy chain homodimer formation hypothesis). Another hypothesis is the misfolding hypothesis. The heavy chains of HLA-B27 misfold in the endoplasmic reticulum and give rise to a pro-inflammatory unfolded protein response (1). However none of these hypotheses can completely explain the mechanism by which HLA-B27 alleles predispose to AS (17).

Although the strength of the association between the HLA-B27 allele and the development of AS is one of the strongest genetic associations with any common disease, only a small percentage of HLA-B27 carriers actually develop AS (16). The risk for developing AS is about 5% in HLA-B27 positive individuals and substantially higher in HLA-B27 positive patient relatives (18). This indicates that other factors, either genetic or environmental, also influence the risk for developing

AS. First of all, different subtypes of HLA-B27 with different strengths of association for AS exist. Additionally, other HLA genes associated with AS are HLA-Bw60, HLA-B38 and HLA-B52. Finally, other non MHC genes are also involved in the susceptibility to AS as revealed by several genome-wide association studies (1, 16). Examples of these are interleukin (IL) 23 receptor, endoplasmic reticulum aminopeptidase 1, IL 1 receptor 2, antrax toxin receptor 2, gene deserts at chromosome 2p15 and 21q22, caspase recruitment domain family member 9, tumor necrosis factor (TNF) (ligand) superfamily member 15 and killer-cell immunoglobulin-like receptor genes (16). There is no evidence of a genetic factor linked to gender or a hormonal factor to give an explanation to the gender differences in AS (3).

1.3.2. Environmental factors

As stated earlier, the susceptibility for AS is largely genetically determined, however substantial evidence is growing that common environmental and pathogenic triggers are also involved (16). Studies with HLA-B27 transgenic rats demonstrate the ubiquitous nature of the involved environmental factor in AS as rats exposed to normal enteric commensal bacteria develop the disease, in contrast to the rats maintained in germ free conditions (19). Also the development of inflammatory bowel disease and the increased gut permeability in some AS patients shows an association between gut and joint inflammation (20). This can be explained by the bidirectional communication between (intestinal) microbiota and the immune system. The microbiota educate the immune response and in its turn the immune system regulates the quantity of microbiota. A role of IL-23 pathways is also possible as it induces local mucosal dysregulation in the ileum of the intestine and IL-17/IL22-dependent enthesitis (21). Next to the microbiota-mediated environmental trigger, another environmental trigger is mechanical stress, which shapes and promotes entheseal inflammation and new bone formation in AS (1, 22). The mechanisms of this relationship is still under investigation. It has been suggested that the extracellular signal-regulated kinase 1/2 plays an important role in mechanotransduction-associated inflammation (23).

1.3.3. AS as an autoimmune disease

AS is historically more known as an auto-inflammatory disease. An auto-inflammatory disease is characterized by mutations in single immunomodulatory genes leading to development of inflammation from excess cytokine production in the absence of clear autoimmunity (22, 24). Examples of pro-inflammatory cytokines that are found in arthritic lesions of AS patients are IL-6 and TNF-α (22). However, the involvement of the immune response is also believed to play a role in AS. Most immune theories about the pathogenesis of AS involve a cell-mediated immune response involving T cells, like the involvement of the IL17/IL23 pathway, the control of nuclear factor-κB activation, amino acid trimming of the MHC antigen presentation and the control of CD8 and CD4 T cell subsets (1, 22). Although autoantibodies are not considered as a classic hallmark of AS, the amount of studies of the involvement of the systemic humoral immune response, with B cells and antibody production, has been increasing enormously (22, 25). First of all, ectopic lymphoid germinal centers together with the presence of plasma cells are found in synovial tissue of AS patients. Also there is proof that the amount of B-cells and plasma cells is raised in the blood

of AS patients (26). Next, it has been demonstrated that AS patients have a greater prevalence of autoantibodies directed against CD74, a class II-associated invariant chain peptide (27). The finding of anti-noggin and anti-sclerostin-containing immune complexes in AS patients confirm the presence of autoimmune mechanisms as well. These immune complexes are believed to be involved in the neo-ossification in AS (28). Taken together, these features suggest more and more a possible role of B cells and antibodies in the AS pathogeneses.

1.4. Diagnosis

Because the basic mechanisms of disease pathogenesis of AS are still poorly understood, the diagnosis and classification remains primarily based on the expertise of the rheumatologists and the presence of symptoms (29).

1.4.1. Classification vs diagnosis

Several criteria have been established to facilitate the diagnosis of AS. The most widely used criteria for classification and diagnosis of AS are the **modified New York criteria (mNYC)** (1984). These incorporate IBP and the radiographic evidence of SI changes, as sacroiliitis is a hallmark of AS with a high prevalence in AS patients (3, 30). With the use of mNYC, a patient can be classified as having definite AS, if the radiological criterion (radiographic sacroiliitis of grade 2 bilaterally or grade 3-4 unilaterally) is associated with at least one clinical criterion (IBP, limitation of mobility of the lumbar spine, or limitation of chest expansion) (30).

Since it takes years from the onset of IBP to develop radiographic sacroiliitis, it can be difficult to make an early diagnosis using radiographic imaging (30). mNYC are therefore not ideal to capture early stages of axSpA or nr-axSpA, as radiographic imaging is necessary. With the emergence of magnetic resonance imaging (MRI) early inflammation could be noted, which is an indication for emerging structural damage, capturing a part of the early radiographic axSpa and nr-axSpA. Criteria that tries to capture both early and established disease, are the **Assessment of SpondyloArthritis International Society** (**ASAS**) classification criteria (**Figure 1**) (29, 31). The ASAS concept divides axSpA into two groups: a group that meets the mNYC AS (the radiographic axSpA) and a second group that does not fit with the mNYC called non-radiographic axSpA (nr-axSpA) (29). A patient can be classified as axSpA in two ways: First by the imaging arm: having a plain film evidence of erosion (like the mNYC) or MRI evidence of SI joint inflammation and one feature of SpA such as uveitis, IBP, family history of SpA. Secondly, by the HLA-B27 arm: being HLA-B27 positive and having two features of SpA (nr-axSpA) (29).

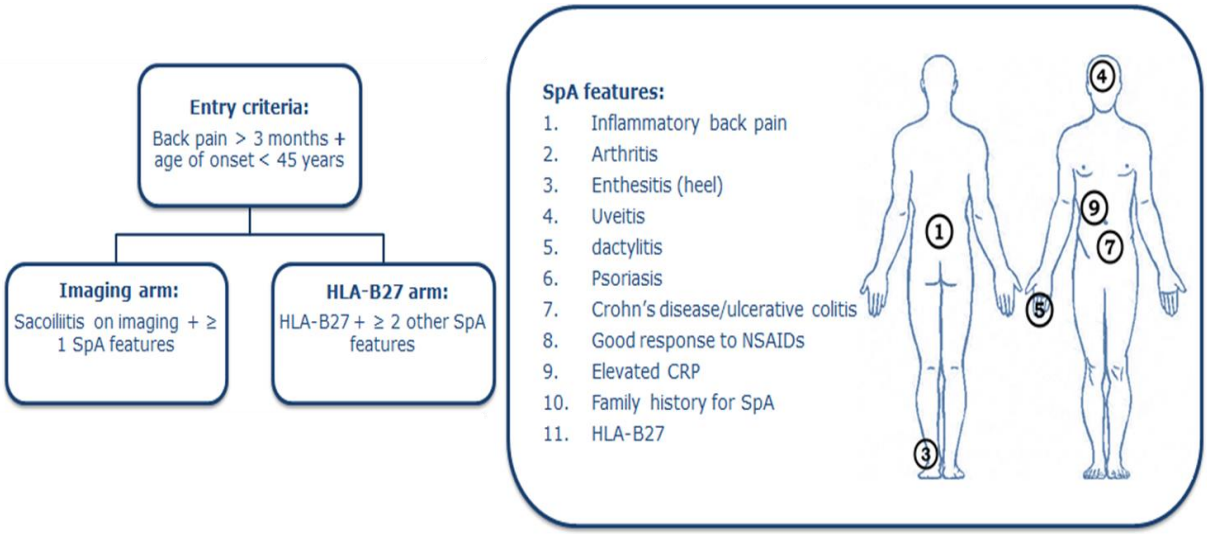

Figure 1: The Assessment of Spondyloarthritis International Society (ASAS) criteria. Criteria to classify axial spondyloarthritis (ax SpA) patients in 2 groups, radiographic (via imaging arm) and non-radiographic (via HLA-B27 arm). Abbreviations: NSAID: nonsteroidal anti-inflammatory drug; CRP: C-reactive protein

The ASAS criteria have a specificity and sensitivity of respectively 84% and 83% (29). This means that approximately 20% of the AS patients will not meet the criteria and be false-negatively classified while 20% of subjects that fulfill the criteria will be false-positives (3). Depending on the expertise of the physician in the field, the diagnosis of AS still largely relies on the presence of radiographic sacroiliitis. This is translated in daily practice: a patient with chronic IBP and radiographic sacroiliitis will be more readily diagnosed as having AS by the physician, in contrast to a patient with only chronic IBP that may be diagnosed as having pre-radiographic axSpA (or nr-axSpA) (30, 32). The imaging arm of the ASAS criteria has thus a high specificity (97%), but a low sensitivity (66%) (29). However radiographic changes appear years after the disease onset of AS, leading to a long delay in diagnosis (30). This diagnosis delay in AS is the longest delay among rheumatologic diseases as it can take seven to ten years before the diagnosis is made in an AS patient (5, 33, 34). Other factors contributing to this delay are: the difficulties in recognizing IBP from chronic back pain, mild-to-moderate symptoms at presentation, absence of a definitive serological diagnostic tests (which is sensitive and specific), low prevalence, slow disease progression, lack of familiarity and knowledge of AS in the general population and among healthcare professionals and a limitation of traditional diagnostic criteria (35). In conclusion, because of the lack of specific serological tests and clear pathognomonic disease characteristics, the diagnosis of AS is delayed.

This delay in diagnosis has several negative consequences, as inflammation in the SI joints and/or other parts of the axial skeleton already occurs in the early stage of the disease, causing disease activity and pain (30, 36). Subsequently, delayed diagnosis of AS causes worse outcomes in disease activity, function, spinal mobility and/or radiographic damage, like painful irreversible damage and stiffening of the spine, as definite diagnosis can lead to clinical improvements and proper management (30, 34). Delay in diagnosis is also associated with poorer treatment responses since the most effective current medications, the class of biologic agents that block TNF-

α, are shown to give a better response in AS patients with short disease duration, when inflammatory burden is the greatest (22, 34). Also, since AS affects the quality of life and induces loss of productivity, due to work disability and sick leave at relative young age, the substantial direct and indirect costs associated with AS result in an average total cost of €9,462 per patient per year in Europe (37). It is thus important to make an early and reliable diagnosis of AS to prevent irreversible long-term consequences and to give a better outcome with the use of biological agents, increasing the patient's quality of life.

1.4.2. Biomarkers for diagnosis

Finding an appropriate biomarker can accelerate the diagnosis of AS, as it is a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (definition of the national institutes of health biomarkers) (38). Currently, only the genetic marker HLA-B27 is commonly used for the diagnosis since it has a high sensitivity (39). However HLA-B27 is not a good diagnostic marker for AS on its own, as it has a low specificity due to its relative high population prevalence (39). Other clinically markers used in the clinic are C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) (39). These markers are elevated in 40-50% of the AS patients, making them not very practical biomarkers for diagnosis, as they are not very sensitive or specific (9, 39).

As blood is easily accessible and can be obtained in large quantities, finding a biomarker in this medium would be very convenient. Differentially expressed proteins are good markers to be discovered in plasma or serum. However sensitivity to measure such a protein marker should be extremely high as such proteins become highly diluted when they are released from their disease targeted organs/tissues into the circulation. Antibodies on the other hand reach higher sensitivity and are very specific to their corresponding antigen target (40). Also when taking the rising indications of the involvement of the humoral immune response in the pathogenesis of AS into account, it is worth to investigate the possibility that antibodies can be good biomarkers for the diagnosis of AS.

1.5. Research approach

Finding other sensitive and specific biomarkers for AS will shorten the delay duration between onset and diagnosis/treatment, preventing the destructive irreversible effects of the disease and increasing the effectivity of the current treatment. The growing information about indications of the involvement of the humoral immune response in AS patients, lead to hypothesizing that an autoantibody will be a biomarker that will improve the delayed diagnosis of AS. This will be investigated by first creating a human cDNA phage display library, that will present a large diversity of possible AS-related antigens. Second, an high throughput screening, serological antigen selection (SAS), will be performed to select only the antigens that bind to autoantibodies present in AS patients serum. Lastly the remaining candidate antigens will be screened for Ab reactivity with the use of phage enzyme linked immunosorbent assay (ELISA) to determine their reactivity in AS serum compared to other rheumatic and healthy controls (HCs).

1.5.1. Human cDNA phage display library

A powerful biomarker discovery tool is the phage display technology. To create a cDNA phage display library, RNA will be isolated from tissues of interest, which are related to the disease, followed by cDNA synthesis (**Figure 2**). The resulting cDNA inserts will be cloned into the M13 filamentous phage phagemid vectors pSPVI-A, pSPVI-B, and pSPVI-C, resulting in fusion of the cDNA to the minor coat protein pVI in three reading frames. This phagemid vector, in combination with a helper phage system, ensures a monovalent display. The cloning results in phage particles that 'display' cDNA products on the surface of their phage coat. The entire phage display library can then be used to screen for other molecules that interact with the proteins or peptides that are exposed on the phage surface.

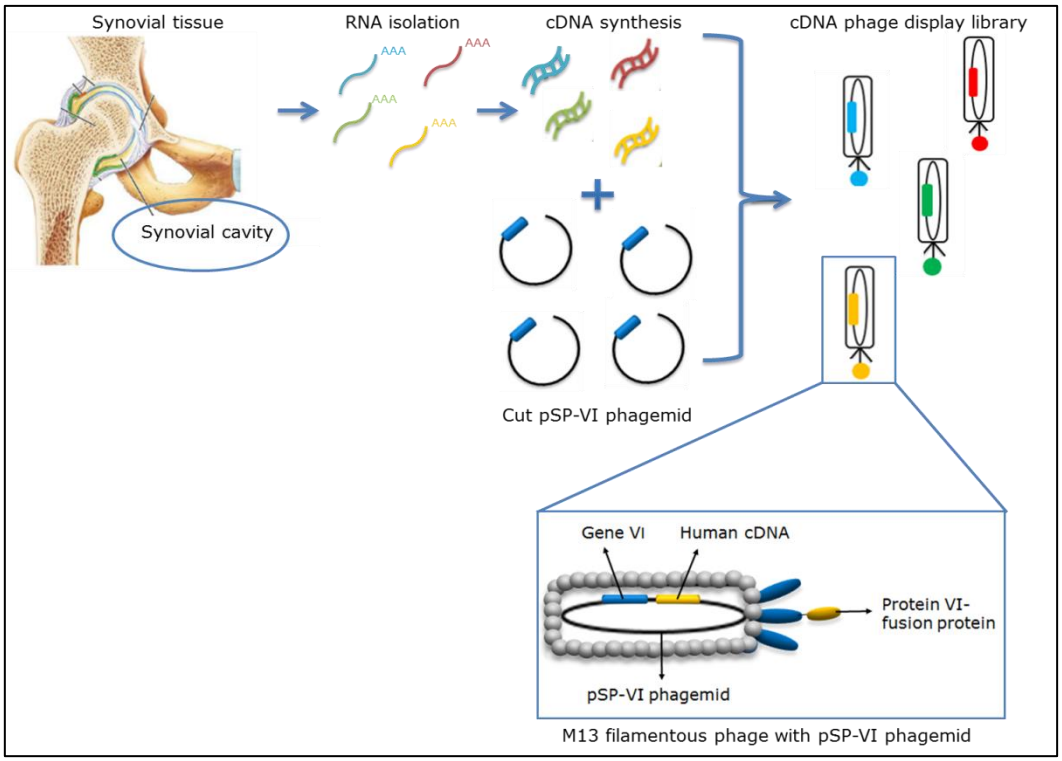

Figure 2: Construction of a cDNA phage display library using M13 filamentous phage. First synovial tissue is collected, followed by RNA isolation. cDNA is created and fused in a pSP-VI phagemid vector. This results in phage particles expressing the cDNA product on the minor coat of protein VI as a fusion product.

1.5.2. Serological antigen selection (SAS)

After the creation of a cDNA phage display library, a selection will be performed on the phage displaying antigens that are reactive against antibodies present in AS serum. This selection will be done using a powerful high-throughput autoantibody profiling technique called serological antigen selection (SAS) (41). By adding pooled patient sera to the cDNA phage display library the formation of antigen-antibody complexes is allowed. Screening several times for these antigen-antibody complexes with patient sera will lead to selective enrichments of the reactive antigens. To ensure specificity of disease-related antigens, a negative selection round will be performed, using sera from a HC pool (or another pool different from the disease pool), eliminating nonspecific reactivity.

1.5.3. Characterization by phage ELISA

With the use of phage ELISA, the reactivity of the identified antigens against antibodies in sera of AS patients, rheumatic controls and HCs will be investigated. The phage ELISA is a sandwich ELISA by which the individual phage particles will be captured by an antibody that binds the major coat protein of phages (**Figure 3**). Subsequently, individual captured phage clones are incubated with individual serum samples of AS patients. In addition, this incubation is also performed with individual serum samples of HCs. Reactive antibodies, present in the serum, bind the antigens expressed on the phage surface. With a secondary anti-human IgG antibody, the degree of reactivity in the serum of AS patients and HC will be measured. Only the antigens that express higher antibody reactivity in serum of the individual AS patients, compared to HC serum will be further characterized in a larger cohort of AS patients, rheumatic controls and HCs.

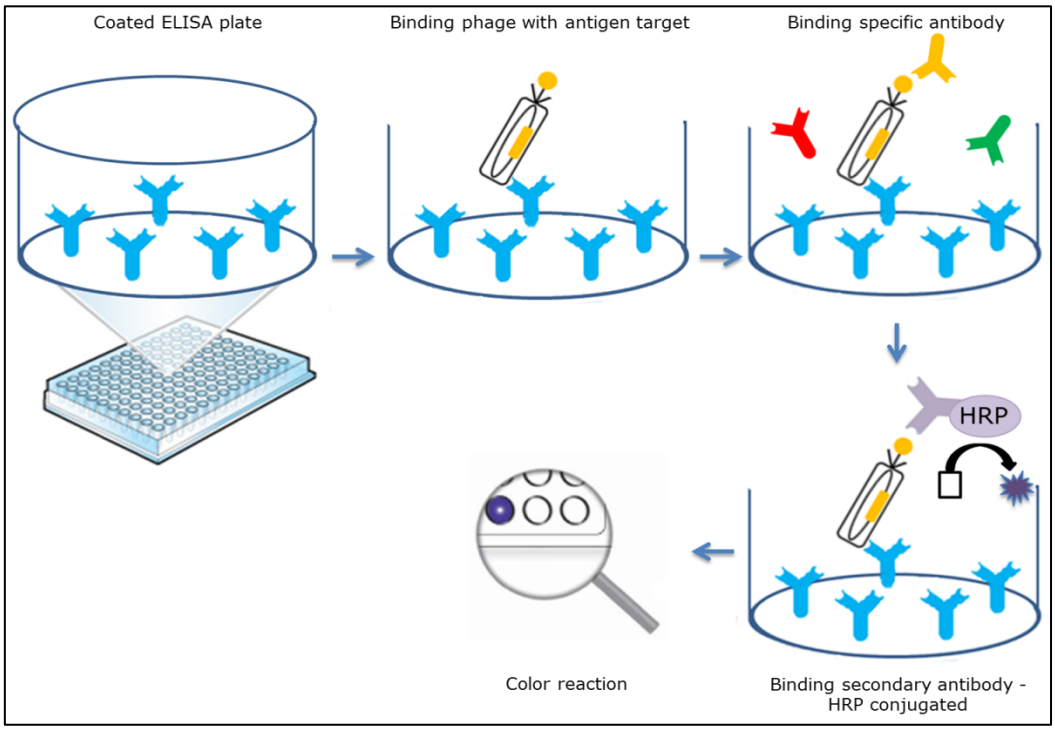

Figure 3: Phage ELISA. The coated ELISA plate binds phage particles, which express specific antigenic targets on their coat. Patient or HC serum containing antibodies is added. Specific antibodies bind to their antigen target, resulting in antibody reactivity. After washing away the nonspecific antibodies, secondary antibody is added. This secondary antibody is coupled to an enzyme that causes an color reaction when adding a chromogenic substrate, making reactivity visible.

2. Materials and methods

2.1. Human AS cDNA phage display library

AS synovial tissue was used for the construction of a cDNA phage display library to obtain a full representation of the heterogeneous antigen composition of AS. This will increase the probability of identifying novel Ab biomarkers specific for AS.

2.1.1. Patients

Synovial tissue of 5 AS patients was acquired for the construction of the human cDNA phage display library (**Table 1**). Three tissues originated from the hip, coming from women. The other 2 tissues were obtained from men's spine. Two patients were treated with the biological agent *Enbrel*, a TNF-α blocker. Another single patient used the disease–modifying anti-rheumatic drug *Sulfasalazine*. The average age of the patients was 54.6 years and 3 patients were HLA-B27 positive.

Table 1: Characteristics of ankylosing spondylitis (AS) patients used for the human ankylosing spondylitis cDNA phage display library.

Patient	Tissue	Gender[a]	Age	HLA-B27[b]	Therapy
AS p01	hip	F	61	+	Enbrel
AS p02	hip	F	69	NA	NA
AS p03	hip	F	51	+	Enbrel
AS p04	spine	M	52	NA	Sulfasalazine
AS p05	spine	M	40	+	Enbrel

[a]Gender: Female (F) and male (M)

[b]HLA-B27 status: positive (+), negative (-), not available (NA)

2.1.2. Construction human AS cDNA phage display library

RNA isolation

Synovial tissue of 5 AS patients was cut in 10 µm sections using a cryostat, LEICA CM 3050 S (*Leica Biosystems, Germany, Nussloch*). Total RNA was isolated from 100 mg tissue using 1 ml Qiazol (*Qiagen, Germany, Hilden*). The tissue was disrupted with a pestle to allow lysis of the cells. Homogenization of the suspension was done by passing 10 times through a 20G needle. The suspension was centrifuged for 15 min at 12,000 g and 4°C after 200 µl chloroform was added. The upper layer, containing the RNA, was transferred into a clean Eppendorf tube and an equal volume of 70% ethanol (EtOH) (*VWR Prolabo, USA, Radnor, PA*) was added. The solution was transferred onto a RNeasy column and the spin column protocol of the RNeasy mini kit (*Qiagen, Germany, Hilden*) was performed according to the manufacturer's instructions. The resulting RNA concentration of the individual samples was measured by the Nanodrop 2000 (*Isogen Life Science, the Netherlands, De Meem*) and samples were stored at -80°C. The quality of the isolated RNA was determined with the Bioanalyzer 2100 (*Agilent Technologies, USA, Santa Clara, California*) using an RNA Nano chip and the Eukaryote Total RNA Nano program following manufacturer's guidelines.

cDNA synthesis

Isolated RNA was converted to double-stranded cDNA using the PV210 oligo dT primer (**Table 2**) and the Maxima H minus double-stranded cDNA synthesis kit (*Thermo Scientific, USA, Waltham, Massachusetts*) according to the manufacturer's instructions. The PV210 oligo dT primer, which was used for first strand cDNA synthesis, was targeted to the edge of the poly-A tail by a VN anchor, where V stands for A, G or C and N for A, T, G or C. This primer also contained a XhoI restriction site for directional cloning afterwards.

Table 2: Characteristics of the PV210 oligo dT primer used for double-stranded cDNA synthesis. V stands for A, or C and N for A, T, G or C

Nr	Sequence	Modification
PV210	*5'gagagagagagagagagaga*ctcgagttttttttttttttttttttt VN 3'	*$(GA)_{10}$*-XhoI-dT_{22}-V(G/C/A)N anchor

After the second strand cDNA synthesis, 10U of T4 DNA polymerase (*Thermo Scientific, USA, Waltham, Massachusetts*) was added and incubated for 10 min at 16°C for the formation of blunt ends. The reaction was stopped by the addition of 0.5 M ethylenediaminetetraacetic acid (EDTA) (*Sigma-Aldrich, Belgium, Diegem*), pH 8.0. Residual RNA was removed by a 10 min incubation at room temperature (RT) with 100 U RNaseI. (*New England Biolabs, USA, Ipswhich, Massachussetts*). Resulting double-stranded (ds) cDNA was purified by phenol/chloroform/isoamyl alcohol (PCI) (*Sigma-Aldrich, Belgium, Diegem*) extraction and EtOH precipitation and was stored at -20°C.

Adaptor preparation

Corresponding with the 5 AS patients, 5 different adaptors were ligated to the ds cDNA inserts, allowing the determination of the patient's tissue from which a specific antigen was originating (**Table 3**). These adaptors have a phosphorylated blunt-ended side and a non-phosphorylated side with an EcoRI overhang. Prior to the ligation, adaptors were prepared by adding NEB buffer 2 (*New England Biolabs, USA, Ipswhich, Massachussetts*) and nuclease-free water to the forward and reverse oligonucleotides (100 µM). The oligonucleotide mixture was incubated for 5 min at 95°C in a heat block, transferred to a container with water at 80°C, which was allowed to cool slowly to RT to ensure formation of the adaptors by annealing of the forward and reverse oligonucleotides.

Adaptor ligation

Nuclease-free water and T4 DNA ligase buffer (*Thermo Scientific, USA, Waltham, Massachusetts*) were added to the precipitated ds cDNA pellet and incubated for 10 min at 37°C. Subsequently, polyethylene glycol (PEG) 4000 (*VWR Prolabo, USA, Radnor, PA)*, the adaptors and T4 DNA ligase (*Thermo Scientific, USA, Waltham, Massachusetts*) were added to the corresponding patient's cDNA and incubated for 3 hrs at 22°C. The T4 DNA ligase was heat inactivated for 20 min at 65°C.

Table 3: Sequences of the 5 adaptors corresponding to the 5 AS patients

Patient	Nr	Sequence	Modification
AS p01	**Adaptor 1**		
	PV230	5'p-*GACGTC***GCGGCCGC**G 3'	*AatII*-**NotI**
	PV231	5'OH-AATTC**GCGGCCGC***GACGTC* 3'	EcoRI overhang-**NotI**-*AatII*
AS p02	**Adaptor 2**		
	PV232	5'p-*GTGCAC***GCGGCCGC**G 3'	*ApaLI*-**NotI**
	PV233	5'OH-AATTC**GCGGCCGC***GTGCAC* 3'	EcoRI overhang-**NotI**-*ApaLI*
AS p03	**Adaptor 3**		
	PV234	5'p-*CCTAGG***GCGGCCGC**G 3'	*AvrII*-**NotI**
	PV235	5' OH-AATTC***GCGGCCGC****CCTAGG* 3'	EcoRI overhang-**NotI**-*AvrII*
AS p04	**Adaptor 4**		
	PV236	5'p-*GTATCC***GCGGCCGC**G 3'	*BciVI*-**NotI**
	PV237	5' OH-AATTC**GCGGCCGC***GTATCC* 3'	EcoRI overhang-**NotI**-*BciVI*
AS p05	**Adaptor 5**		
	PV238	5'p-*AGATCT***GCGGCCGC**G 3'	*BgIII*-**NotI**
	PV239	5' OH-AATTC**GCGGCCGC***AGATCT* 3'	EcoRI overhang-**NotI**-*BgIII*

cDNA fractionation

cDNA inserts were fractionated according to their size using agarose gel electrophoresis in order to optimize the insert:vector ratio during the cDNA library ligation. The 5 different adaptor ligation mixtures representing the 5 AS patients were pooled and loaded on a agarose gel (0.8%). After electrophoresis, the gel was cut into 6 different fractions, based on the smart ladder (*Eurogentec, Belgium, Liege*) (**Table 4**). Each of these different gel fragments was purified using Nucleospin columns (*Macherey-Nagel, Germany, Düren*) and the DNA concentration of each fraction was determined with the Nanodrop 2000.

Table 4: Different fractions based on size (base pair (bp)) for the cDNA library ligation

Fraction	Size (bp)
A	200–400
B	400–800
C	800–1600
D	1600–3200
E	3200–6400
F	>6400

Vector restriction digestion

Prior to ligation of the cDNA inserts into the phagemid vector, the vector DNA of pSPVI-A, pSPVI-B and pSPVI-C, each coding a different reading frame, was digested using the restriction enzymes EcoRI and XhoI in NEB cutsmart buffer (both enzymes and buffer are from: *New England Biolabs, USA, Ipswhich, Massachussetts*). These three mixtures were incubated for 3 hrs at 37°C, whereupon the enzymes were heat inactivated for 20 min at 65°C. These reactions were loaded on an agarose gel electrophoresis (1%) and fragments of 3600 base pair (bp) were purified according to the Nucleospin protocol.

cDNA library ligation

Equal amounts of EcoRI and XhoI digested vectors were mixed to a concentration of 50ng/µl. For each cDNA fraction, EcoRI/XhoI digested pSPVI-A/B/C vector mixture was added at a 3:1 (insert:vector) molar ratio. The cDNA inserts were ligated into the three pSPVI vectors, by adding T4 DNA ligase buffer and T4 DNA ligase (5U/µl) and incubating the mixture overnight at 16°C. Incubation for 5 min at 70°C inactivated the T4 DNA ligase. Subsequently, the six ligation mixtures were pooled and purified via PCI extraction and EtOH precipitation. After the EtOH was air dried, the pooled ligation mixture was dissolved in 20 µl TE buffer (10 mM Tris/hydrogen chloride, 1 mM EDTA, pH8,0) and stored at -20°C.

TG1 electroporation

High efficiency transformation of the cDNA ligation mixture to *Escheria coli (E. coli*) TG1 electrocompetent cells was achieved by electroporation. The ligation mixture (1µl) was added to the electrocompetent cells (20µl), briefly stirred and transferred to a 1 mm electroporation cuvette. The electroporation was done with the BioRad µpulser (*Biorad, Belgium, Brussels*) under the following conditions: a pulse of 10 µF, 600 Ohm and 1.8 kV. Cells were recovered by adding recovery medium, followed by incubation for 1 hr at 37°C, while shaking at 250 rpm. The resulting transformed *E.coli* cells were plated on 2xTY-ampicillin (*Roche, Switzerland, Basel*)-glucose (20%) (*VWR, USA, Radnor, PA*) ((2xTYAG) plates and incubated overnight at 30°C.

2.1.3. Optimization experiments

Testing adaptors

The same five adaptors were prepared again as previously mentioned. A test vector (pLenti vector SYN-GCaMP3) was cut with EcoRV (*New England Biolabs, USA, Ipswhich, Massachussetts*) to create blunt ended fragments of 1421 bp. Ligation of these test fragments with the old (5) and new (5) adaptors was performed under the same conditions as previously described. The resulting ligation products were phosphorylated by adding T4 DNA ligase buffer and T4 polynucleotide kinase (*Thermo Scientific, USA, Waltham, Massachusetts*). pSPVI-A vector was cut with EcoRI and dephosphorylated using rAPid alkaline phosphatase (*Roche, Switzerland, Basel*) and rAPid alkaline phosphatase (*Roche, Switzerland, Basel*). This dephosphorylated EcoRI-cut pSPVI-A vector was ligated with each adaptor-test fragment product (10) under the same conditions as the cDNA library ligation. As a negative control, the empty pSPVI-A vector was used and an EcoRI cut vector fragment (vector pCAG mCX3CR1; 1104 bp), ligated into the psPVI-A vector, was used as a positive control. These ligation reactions were transformed into TOP 10 chemically competent *E. coli* cells (*Thermo Scientific, USA, Waltham, Massachusetts*) according to manufacturer's guidelines.

Colony PCR

Colony PCR was performed on 74 randomly selected bacterial colonies, resulting from the *TG1* electroporation, to determine the size of the inserts of the human AS cDNA phage display library. Colonies were added with Taqbuffer (*Roche, Switzerland, Basel*), dNTP mixture, forward primer gene VI (100µM), reverse primer pSPVI (100µM) (both primers are from *IDT, Integrated DNA technologies, USA, Coralville, Iowa*) and Taq DNA polymerase (*Roche, Switzerland, Basel*). PCR amplification was performed under the following conditions: 10 min at 92°C, followed by 20 sec at 94°C, 30 sec at 58°C, 3.5 min at 68°C repeated for 30 times, finished by 10 min at 68°C in a Biorad T100 Thermo cycler (*BioRad, Belgium, Brussel*). Amplified PCR products were loaded on a 2% agarose gel to separate the cDNA based on size, and compared to a 100 bp DNA ladder (100bp L) (*Thermo Scientific, USA, Waltham, Massachusetts*).

2.2. Screening human RA cDNA phage display library for Ab reactivity in AS patients

In anticipation of an human AS cDNA phage display library with sufficient diversity, an existing human RA cDNA phage display library was screened for antibody reactivity against pooled AS plasma using the powerful, high-throughput approach of SAS, followed by phage ELISA.

2.2.1. Patients

For the screening of the human RA cDNA phage display library, plasma samples from 10 AS patients were selected (**Table 5**). Half of the patients were men and the average age was 40.6 years. Almost all patients were HLA-B27 positive: 8/10. The patients were selected based on the duration between diagnosis and sampling (maximum 2 years) and the duration between occurrence of clinical symptoms and sampling (maximum 3 years).

Table 5: Characteristics of AS patients used for screening the human RA cDNA phage display library using SAS

Patient	Gender	Age (years)	HLA-B27	Disease duration (years)
AS-RA p01	F	49	+	2
AS-RA p02	M	26	+	0
AS-RA p03	F	23	+	0
AS-RA p04	M	55	+	3
AS-RA p05	M	46	+	3
AS-RA p06	M	52	-	2
AS-RA p07	F	53	+	1
AS-RA p08	F	37	+	3
AS-RA p09	F	31	+	2
AS-RA p10	M	34	-	2

2.2.2. Characterization human RA phage display library

The human RA cDNA phage display library was characterized to determine its composition, which should have a broad range of different inserts and an adequate representation of the 3 different pSPVI vectors and the 3 different RA patients, it was constructed from. This is to ensure, that a cDNA library of high diversity can be used for the screening for AS specific antibody reactivity.

Colony PCR

A sample of the human RA phage display library (TG1 glycerol stock with a total diversity of 9.27 x 10^6 colony forming units (cfu)) was plated to single colonies on 2xTY-AG petri dishes and grown overnight. Colony PCR was performed on 192 randomly selected bacterial colonies as previously described. Ninety-six amplified cDNA products were purified from the agarose gel according to the GFX protocol (*GE Healthcare, United Kingdom, Little Chalfont*).

Cycle sequencing

A Sanger cycle sequencing reaction was performed to identify the 96 inserts, which were acquired from the colony PCR and to determine the representation of the different vectors and the different RA patients. To each PCR product, Big dye buffer, big dye sequence mix (both are from *Thermo Scientific, USA, Waltham, Massachusetts*) and forward primer gene VI (2pmol/µl) were added. The following conditions were used for DNA amplification: 30 sec at 96°C, followed by 10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C repeated for 25 cycles in a Biorad T100 Thermo cycler. Purification of the sequencing products happened by using self-made Sephadex G50 spin columns (*GE Healthcare, United Kingdom, Little Chalfont*), followed by dissolving in highly deionized formamide (*Thermo Scientific, USA, Waltham, Massachusetts*). The sequences were analyzed with the ABI Prism 310 Genetic analyzer (*Thermo Scientific, USA, Waltham, Massachusetts*).

2.2.3. Phage ELISA

The human RA cDNA phage display library was screened for AS specific antibodies with the SAS procedure, resulting in the identification of 67 unique antibody targets. These different targets were tested by ELISA for antibody reactivity in the HC plasma pool. Antibody targets that showed antibody reactivity in the HC pool were excluded. Antibody reactivity against the remaining targets was tested in individual plasma samples of AS patients and HCs by phage-ELISA.

Phage production

Phage particles displaying each individual SAS selected antibody targets, were produced. Bacteria containing the phagemid encoding the antibody target of the selected phage clones were grown overnight from a glycerol stock on minimal plates (10x M9 salts (sodium hydrogen phosphate , monopotassium phosphate , sodium chloride (NaCl), ammonium chloride; all from *VWR, USA, Radnor, PA*), bacto agar (*Becton Dickenson, USA, Franklin Lakes, New Jersey*), 20% glucose, 1M magnesium sulfate heptahydrate (*VWR, USA, Radnor, PA*), 1M calcium chloride dihydrate (*VWR, USA, Radnor, PA*), 1M thiamine hydrochloride (*Sigma-Aldrich, Belgium, Diegem*)) with ampicillin. For each clone, an isolated bacterial colony was picked and cultured overnight at 250 rpm and 37°C in a falcon tube (*Greiner Bio-One, Belgium, Vilvoorde*) containing 2xTYAG medium. This overnight culture was grown further in an Erlenmeyer containing 2xTYAG to an optical density (OD) of 0.5 at 600nm, measured with the Ultrospec 10 cell density meter (*GE Healthcare (Amersham Biosciences), United Kingdom, Little Chalfont*). By adding *M13K07* helper phage (*GE Healthcare, United Kingdom, Little Chalfont*) at a ratio of 20:1 helper phage to bacteria, the bacteria were infected for 30 min at 37°C in a water bath. After centrifugation, using Sigma 4K-15 (*Sigma-Aldrich, Belgium, Diegem*) for 15 min at 4,000g, the resulting pellet was mixed with 2xTY-A Kanamycin sulphate (*Gibco, Thermo Scientific, USA, Waltham, Massachusetts*) (2xTYAK) and grown overnight at 30°C, shaking at 250 rpm in Erlenmeyers. The cells were centrifuged for 15 min at 4,000g and the supernatant (SN), containing the phage particles, was transferred to a falcon tube containing one fifth volume 20 % PEG 6000 (*Carl Roth, Germany, Karlsruhe*) and 2.5M NaCl (*VWR Prolabo, USA, Radnor, PA*) (PEG/NaCl). This precipitation (1hr on ice) was followed by centrifugation 15 min at 4,000g. The acquired pellet was dissolved in phosphate buffered saline (PBS) (potassium hydrogen phosphate (*Merck, USA, Billerica, Massachusetts*), sodium phosphate dibasic dehydrate (*VWR, USA, Radnor, PA*), NaCl, pH: 7.4). Remaining bacteria in the phage precipitate were removed by centrifugation for 5 min at 4,000g. One fifth PEG/NaCl was added to the supernatant and incubated on ice for 20 min. After centrifugation for 15 min at 4,000g, the pellet, containing purified phage particles, was dissolved in PBS + 10% glycerol (*VWR, USA, Radnor, PA*), aliquoted and stored at -80°C.

Titer determination

A titer determination was performed to measure the concentration of the purified phage clones. Ninety-six-well flat-bottomed ELISA plates (*Greiner Bio-One, Belgium, Vilvoorde*) were coated overnight at 4°C with 5 µg/ml anti-M13 monoclonal antibody (*VWR, USA, Radnor, PA*) in coating buffer (disodium carbonate (*VWR, USA, Radnor, PA*), sodium hydrogen carbonate (*Merck, USA, Billerica, Massachusetts*), pH 9.6). Coated plates were washed 2x5 min. with PBS, while shaking at

RT. Blocking with 5% PBS-Marvel (*Premier Foods, England, St Albans*) (PBS-M) happened for 2 hrs at 37°C and 100 rpm. Subsequently, the plates were washed 3x5 min. with 0.1% PBS-Tween (*Merck, USA, Billerica, Massachusetts*) (PBS-T) and 1x5 min. with PBS. The selected phage clones were incubated for 2 hrs shaking at RT, at four different dilutions: 1/10E5, 1/3*10E5, 1/10E6 and 1/3*10E6 cfu/ml, together with a phage of known concentration (1.27x10E13 cfu/ml) at eight different dilutions: 2,54xE8, 2.03x10E8, 1.52x10E8, 1.01x10E8, 5,07x10E7, 2,54x1E7, 1,27x10E7 and 6,34x10E6 cfu/ml. Secondary antibody (1/5000), horseradish peroxidase (HRP)-labeled anti-M13-antibody(*VWR, USA, Radnor, PA*) in PBS-M was added after washing and incubated for 1 hr at RT and 100 rpm. Plates were washed, followed by the addition of 1-step ultra 3,3',5,5' tetramethyl-benzidine dihydrochloride (TMB) (*Perbio Science, Belgium, Diegem*) and kept in the dark for 9 min. The color development was stopped with 1.8N sulfuric acid (H_2SO_4) (*VWR, USA, Radnor, PA*) and the optical densities were measured with a TECAN, hydrospeed microplate reader (*Switzerland, Männedorfl*) at 450nm after 3 seconds of shaking.

Phage ELISA

Phage ELISA was used to determine Ab reactivity against phage clones with a specific antigen. ELISA plates were coated overnight at 4°C with 5µg/ml anti-M13 monoclonal antibody in coating buffer. The coated plates were washed 2x5 min with PBS. Next, the plates were blocked with 5% PBS-M for 2 hrs at 37°C, shaking (100 rpm). Plates were washed 3x5 min with 0.1% PBS-T and 1x5 min with PBS. The chosen phage clones were added at a titer of $7x10^{11}$ cfu/ml and incubated for 1 hr at 37°C and subsequently 30 min shaking at RT. The serum samples (diluted 1/100 in 5% PBS-M) were added after washing and incubated for 1 hr at 37°C, followed by 30 min at RT, shaking. Plates were washed and the secondary antibody HRP-labeled goat anti-human IgG-Fc, cross-adsorbed (*Bethyl (IMTEC), Belgium, Antwerp*) (diluted 1/50 000 in 5% PBS-M) was administered and incubated for 1 hr at RT. 1-step ultra TMB was added, after washing, and the plates were kept in the dark for 11 min. The reaction was stopped with the addition of 1.8N H_2SO_4. Optical density was measured in a TECAN, hydrospeed microplate reader after 3 sec shaking at 450 nm.

Each phage clone was tested in duplicate in a single ELISA experiment and experiments were repeated twice, independently. For each serum sample, the background reactivity against empty phage particles was tested. The average, standard deviation (SD) and the coefficient of variance (CV) were calculated. If the CV was higher than 20%, extra measurements were performed. The ratio of the signal of specific phage vs empty phage was calculated as well as the subtraction of the signal of the empty phage from the specific phage. For each phage, a cut-off value was determined at the average + 3x SD of a population of non-responders. A signal was considered positive when its background subtracted value was above this cut-off.

3. Results

3.1. Human AS cDNA phage display library

To create a human AS cDNA phage display library, RNA was isolated out of synovial tissue of 5 AS patients. The isolated RNA had a concentration ranging between 978 and 3752 ng/µl and the A260/280 and 260/230 ratio were as expected for pure RNA (**Table 6**). RNA from spinal tissue had a higher concentration than the RNA isolated from hip tissue.

Table 6: RNA concentration of the 5 synovial tissues isolated from 5 AS patients with the 260/280 and 260/230 ratio

Sample ID	Tissue origin	Nucleic acid conc. (ng/µl)	A260/280	260/230
AS p01	Hip	977.8	2.08	1.95
AS p02	Hip	3752.5	2.09	2.22
AS p03	Hip	1182	1.98	2.3
AS p04	Spine	2463.4	2.06	2.22
AS p05	Spine	1846.3	2.05	2.16

After the cDNA synthesis and addition of the adaptors, the cDNA inserts were fractionated according to size using agarose gel electrophoresis. The gel showed a smear of different cDNA sizes, indicating a high range of different cDNA sizes (**Figure 4**).The intense band at the bottom of the gel represents the unligated adaptor fragments.

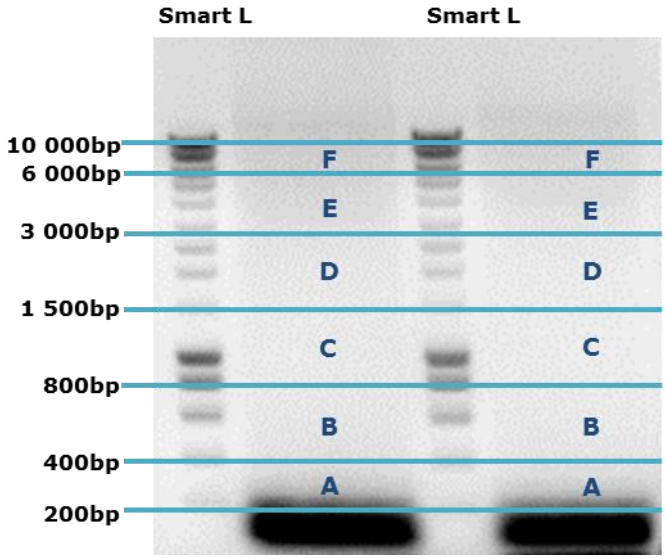

Figure 4: Fractionation of the AS cDNA inserts according to size (in base pair (bp)). The smartladder (Smart L) as reference for size. Six different fractions (A-F) were used for the vector:insert optimization of the ligation.

After isolating the cDNA in different fractions from the gel, the concentration was measured. The concentration was similar for all the fractions, but did not have a very high value (**Table 7**). The 260/230 ratio was very low for all the fractions (chemical contamination).

Table 7: DNA concentration of the 6 different fractions of the cDNA derived from 5 AS patients

Fraction	Nucleic acid conc. (ng/µl)	A260/280	260/230
Fract A	8.5	1.71	0.11
Fract B	6.3	1.77	0.21
Fract C	6.9	1.69	0.07
Fract D	6.4	2.39	0.09
Fract E	4.7	2.22	0.03
Fract F	12.1	1.55	0.13

After electroporation 1 µl of our constructed human AS cDNA phage display library the resulting diversity was **2.5×10^3 cfu**. If we electroporated our entire human AS cDNA phage display library (consisting of 50 µl purified DNA) the diversity would have been **1.25×10^5 cfu**. This diversity is too low to find rare transcripts, which could be relevant in AS pathogenesis. As all steps were believed to be executed properly, we subsequently tested the electrocompetent TG1 cells, the adaptors, the quality of the RNA and the vector-insert ratio.

3.1.1. Optimization

Electrocompetence TG1 *E. Coli* cells

First, the competence of the TG1 electrocompetent cells used for the construction of the AS library was compared to a previous batch of TG1 cells (used for the RA library) of which is known that they work efficiently in an electroporation. Electroporation of the AS cDNA library was repeated with both 'old' and 'new' TG1 cells and a positive control (pUC 19 vector). There was no difference in resulting cfu between the old and new TG1 cells for both the ligation mixtures and the positive control. The positive control gave visibly more cells than the ligation mixtures as 10^{10} cfu were obtained with the positive control in contrast to the 10^3 cfu of the ligation mixtures (**Table 8**). As the positive control gave the number of cfu indicated by the manufacturer, it can be concluded that there was nothing wrong with the TG1 cells, but probably with the ligated cDNA library.

Table 8: The electrocompetence of TG1 *E. coli* cells resulting in no difference between old and new cells. The amount colony forming units (cfu) was counted after electroporation of the AS cDNA library and the positive control (pUC 19 vector).

	New TG1 cells	Old TG1 cells
cDNA products	10^3 cfu	10^3 cfu
pUC 19 vector (positive control)	10^{10} cfu	10^{10} cfu

cDNA insert AS

Since the TG1 cells showed a good electrocompetent capacity, we next controlled the cDNA inserts of a selection of library clones. In order to test these cDNA products, a colony PCR was performed on 74 bacterial colonies.

A 100bp L was used for sizing the inserts on an agarose gel. Out of the 74 colonies that were tested, 55 were successfully amplified using PCR, given a success rate of 74.3% (**Figure 5**). Empty pSPVI vectors were visible on the gel as a 300 bp PCR product. Almost half of the clones (47%) contained an empty pSPVI-vector without cDNA insert (**Figure 6**). The remaining 53% of bacterial colonies contained cDNA inserts with a size ranging from 100–1000 bp as their PCR products size ranged from 400–1300 bp. The majority of the inserts were 300 bp long (PCR product of 600 bp) and only a low percentage bacterial colonies contained a long insert.

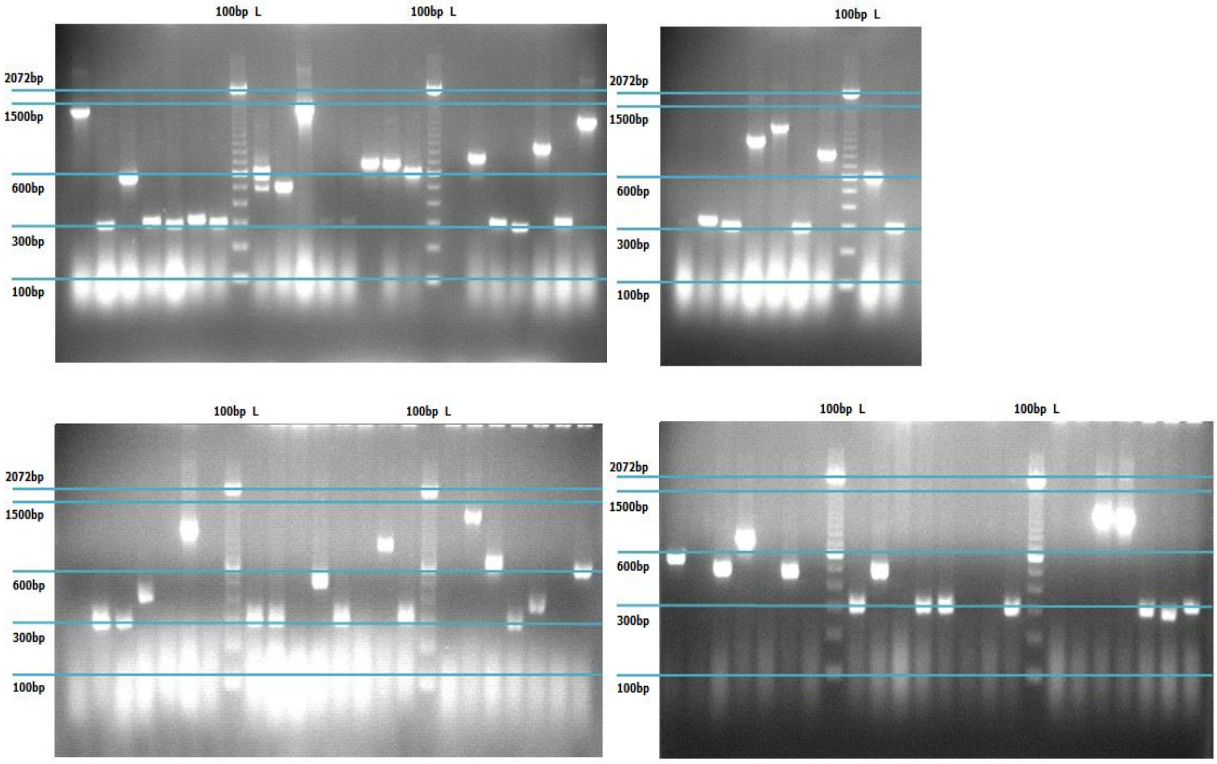

Figure 5: Colony PCR of the cDNA inserts from the constructed human ankylosing spondylitis cDNA phage display library with the majority containing an empty pSPVI vector. The 100 base pair ladder (100bp L) was used as reference for size in bp and marked on the figure. 74 randomly selected bacterial colonies were amplified and visualized on gel resulting in 74.3% success rate. The empty vectors are visible at 300bp on gel.

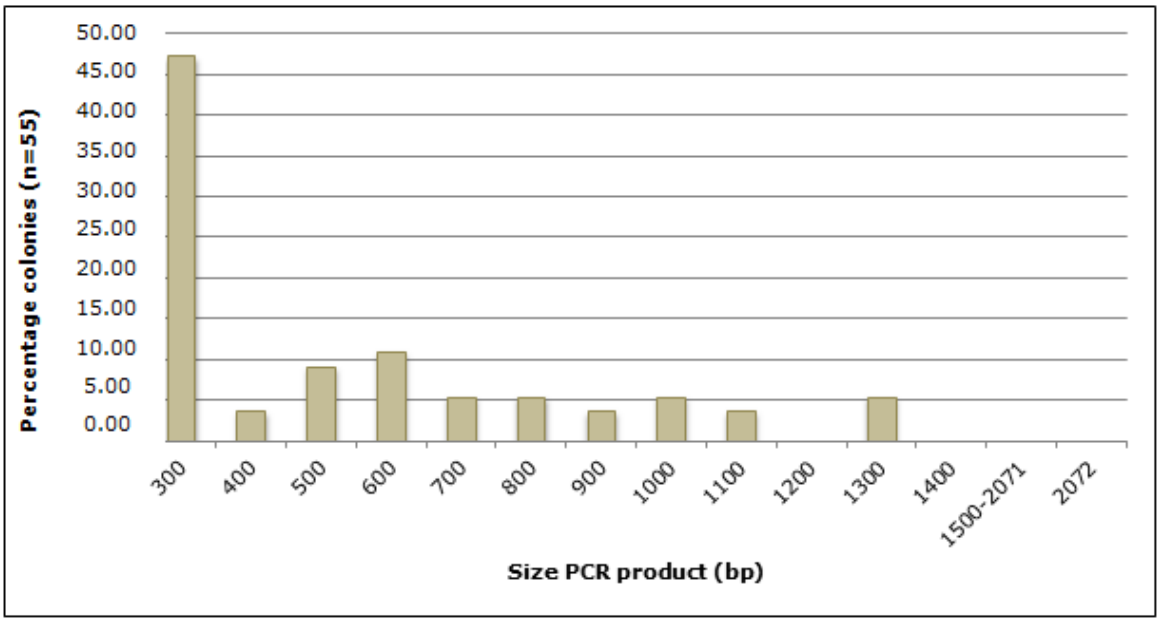

Figure 6: Percentage of the different product sizes of the colony PCRs (n=55) of the cDNA inserts from the human AS cDNA phage display library. Empty vectors have a product size of 300 base pairs (bp). The cloned inserts have a size ranging from 100–1000 bp (400 – 1300 bp PCR products).

From these results, it can be concluded that the cDNA products were not ligated correctly into the vector since we obtained such high percentage of empty vectors. Subsequently, it was tested whether the adaptors were causing the problem. However, a new batch of adaptors was prepared and compared to the old batch, not resulting in a significant change.

Quality isolated RNA of AS synovial tissue

The quality of the total RNA isolated from the synovial tissue of the five AS patients was examined using the Agilent bioanalyzer. Ribosomal RNA (rRNA) consists approximately 80% of the total RNA isolated from cells. The size of the peaks of the main rRNA species, 18S and 28S, give a good indication of the integrity of the total RNA via the RNA integrity number (RIN). The five RNA samples of the AS patients do not have very clear rRNA peaks and the baseline between the marker peak and 18S is very disturbed, indicating that the quality of the RNA is not very high and even highly degraded (**Figure 7**). This in contrast to the RNA control, which has clear ribosomal peaks and a flat baseline between the marker peak and 18S. This difference is also represented by the RIN values: the RNA control has a high RIN value compared to the RNA of the AS samples. There was also a difference between the tissues that were derived from the spine (AS p04-05) and the tissues derived from the hips (AS p01-03). The spinal tissues were more degraded than the hip tissues. Overall, this can be a reason why the insert ligation was not optimal.

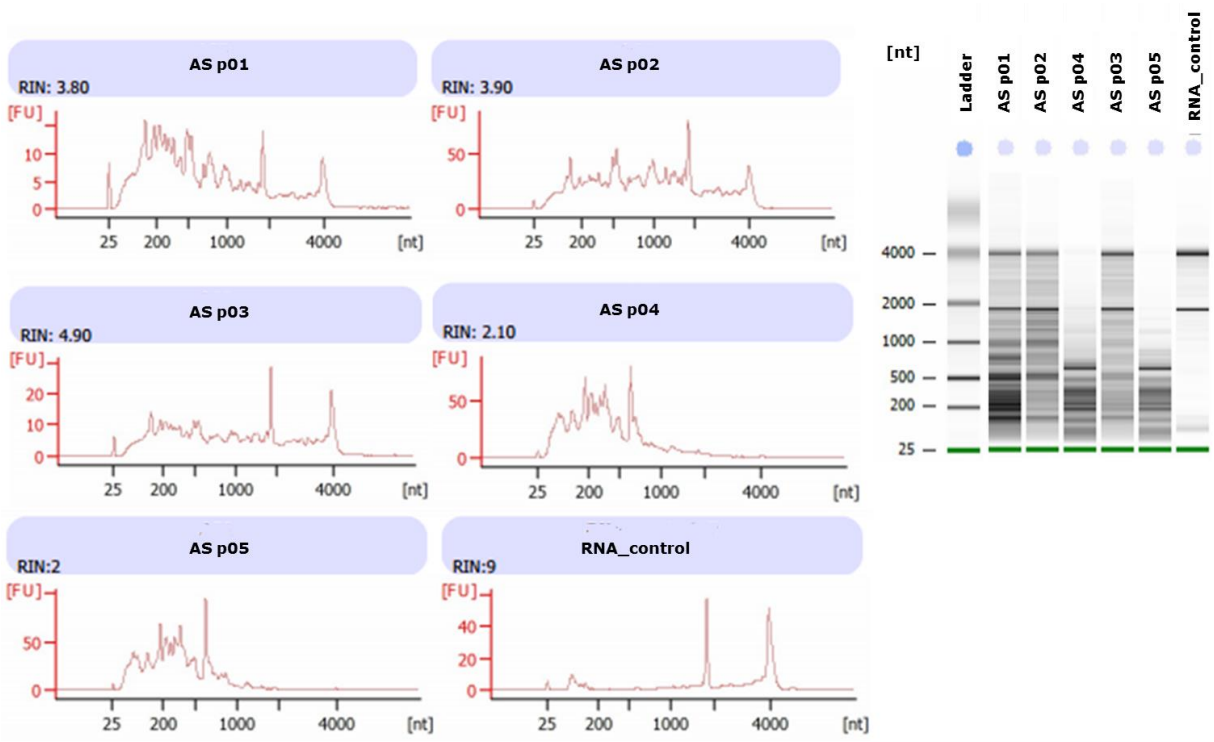

Figure 7: Result bioanalyzer of the 5 RNA samples of the 5 AS patients, showing highly degraded RNA. A RNA_control sample was used as a comparison. The x-axis represents the nucleotide sizes (nt) of the different fragment. The y-axis displays the fluorescence signal in fluorescence units (FU). Next to the marker peak and the two ribosomal peaks (18S and 28S) is the RNA integrity number (RIN) value also stated, as an indication of the quality of the RNA.

3.2. Screening human RA cDNA phage display library for Ab reactivity in AS patients

As the construction of a diverse human AS cDNA phage display library still required further optimization, the human RA cDNA phage display library was used to screen for antigen targets with AS sera.

3.2.1. Characterization human RA cDNA phage display library

Before the RA cDNA phage display library was used in the screening for antigen targets with AS sera, the composition of the library was sampled to ensure a broad range of different cDNA inserts. A high diversity cDNA library increases the chance to find a specific AS antigen target.

cDNA insert size

First, the size of some inserts was determined with colony PCR. The empty pSPVI vector gave a PCR product of 300 bp. Out of the 192 bacterial colonies that were picked, were 157 PCR reactions successful, giving a success rate of 81.8% (**Figure 8**) (**Supplement 1: Colony PCR of the inserts of the human RA cDNA phage display library**).Only a small portion of the bacterial colonies did not contain an insert. The other bacterial colonies presented a high variety of different sized cDNA inserts.

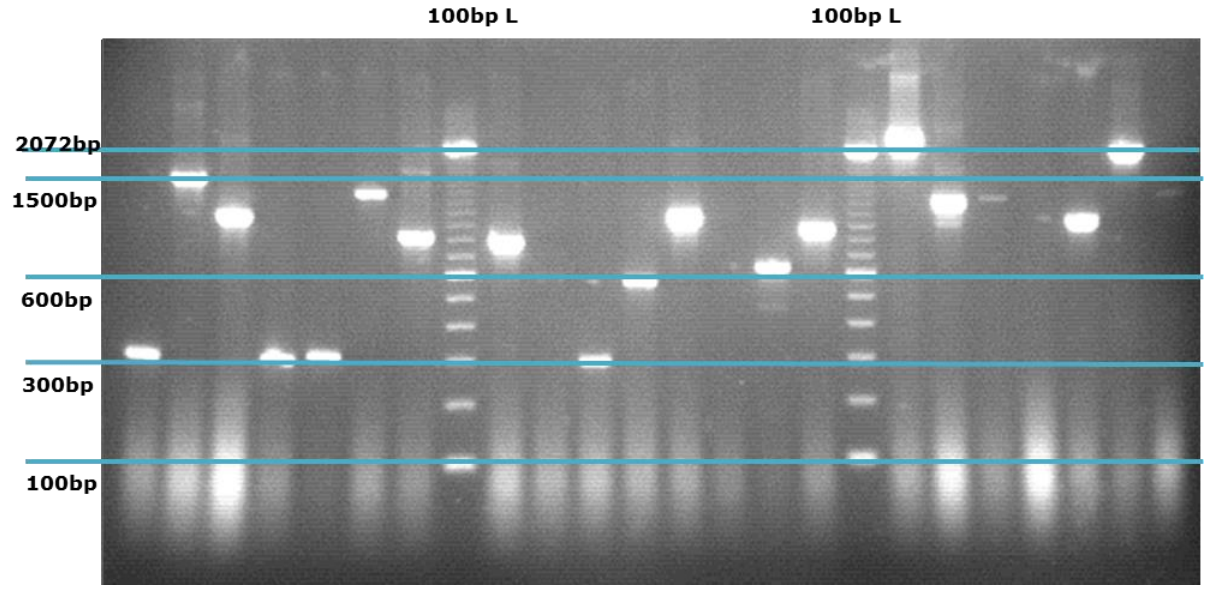

Figure 8: Colony PCR of 22 cDNA inserts from the human RA cDNA phage display library containing a high diversity of different sized cDNA inserts. The 100 base pair ladder (100bp L) was used as reference for size in bp and marked on the figure. The overall success rate of all the colony PCRs was 81.8% success rate. The empty vectors are visible at 300 bp on gel.

Specifically, only 18.5% of the successful PCR reactions showed empty vectors (Figure 9). The other 81.5% had a diverse distribution of different insert sizes, ranging from 100-1800 bp, as showed by PCR products size ranging from 400–2100. This suggests that the human RA cDNA phage display library has a broad range of different cDNA.

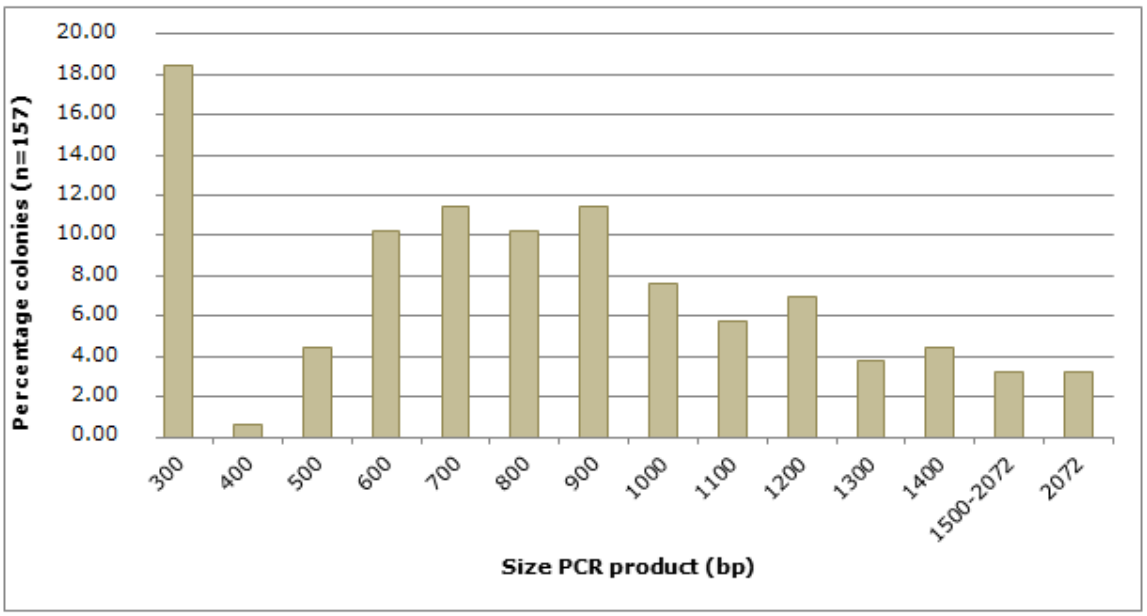

Figure 9: Percentage of the different product sizes of the colony PCRs (n=157) from the human rheumatoid arthritis cDNA phage display library with a high diversity of different sized inserts, ranging from 100–1800 base pairs (bp) (400–2100 bp PCR products). Only 18.5% of the bacterial colonies contained an empty vector (product of 300 bp).

Of the 157 successfully amplified PCR products, 96 were sequenced in using cycle sequencing to determine the distribution of the pSPVI vector, the distribution of the three RA patients, the identity of the insert and the reading frame of the cDNA insert.

Vector and patient distribution

Of the 96 cycle sequencing reactions, 70 reactions succeeded. The three different vectors are distributed more or less equally with 26% pSPVI-A vector, 34% pSPVI-B vector and slightly more pSPVI-A vector with 40% (Figure 10**A**)**.** Also the patients distribution was evenly: 37% of the cDNA originated from RA-patient 1, 31% from RA-patient 2 and 32% from RA-patient 3 (**Figure 10B**).

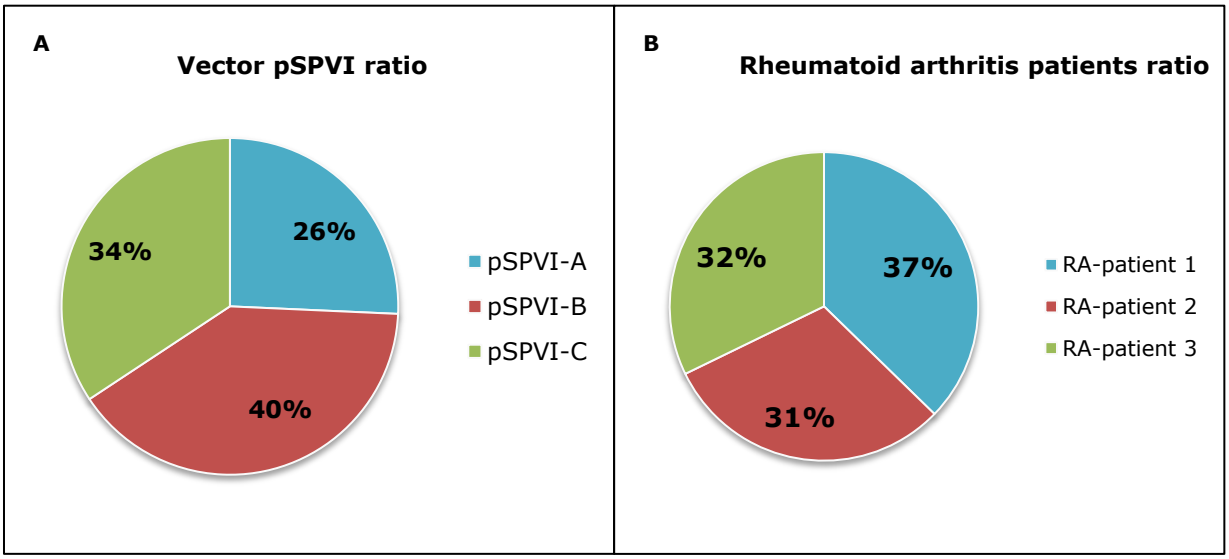

Figure 10: Distribution of vectors and patients in the human RA cDNA phage display library. **A:** Distribution of the three different vectors: pSPVI-A/B/C **B:** Distribution of the three different RA patients used to construct the human RA cDNA phage display library: RA-patient-1, 2, 3

Identification cDNA inserts

Only 17% of the 70 vectors did not contain an insert, indicating the high quality of the cDNA phage display library (**Figure 11**). Of the insert containing clones, the insert typed and the location of the gene fusion were examined. Insert sequences were aligned against human transcript and genomic sequences using nucleotide BLAST (basis local alignment search tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi). For each insert containing clone (n=58), the full length transcript was retrieved from the NCBI database. The start of the insert could be attributed to the 5' or 3' untranslated region (UTR), the coding region, genomic DNA or ribosomal RNA. 30% (n=21) of all inserts were allocated to the 5'UTR of a protein coding transcript, 17% (n=12) to the 3'UTR and 23% (n=15) to the coding region. 7% (n=5) of the inserts were found to originate from rRNA and 6% (n=4) came from genomic, usually intronic sequences.

When the insert was attributed to the 5'UTR or coding region, we also determined for which percentage of inserts the geneVI was in frame with the canonical coding sequence. Eight of the 5'UTR allocated inserts (38%) and 8 of the coding region allocated inserts (50%) were in frame with gene VI, each corresponding to 13.8% of the total library.

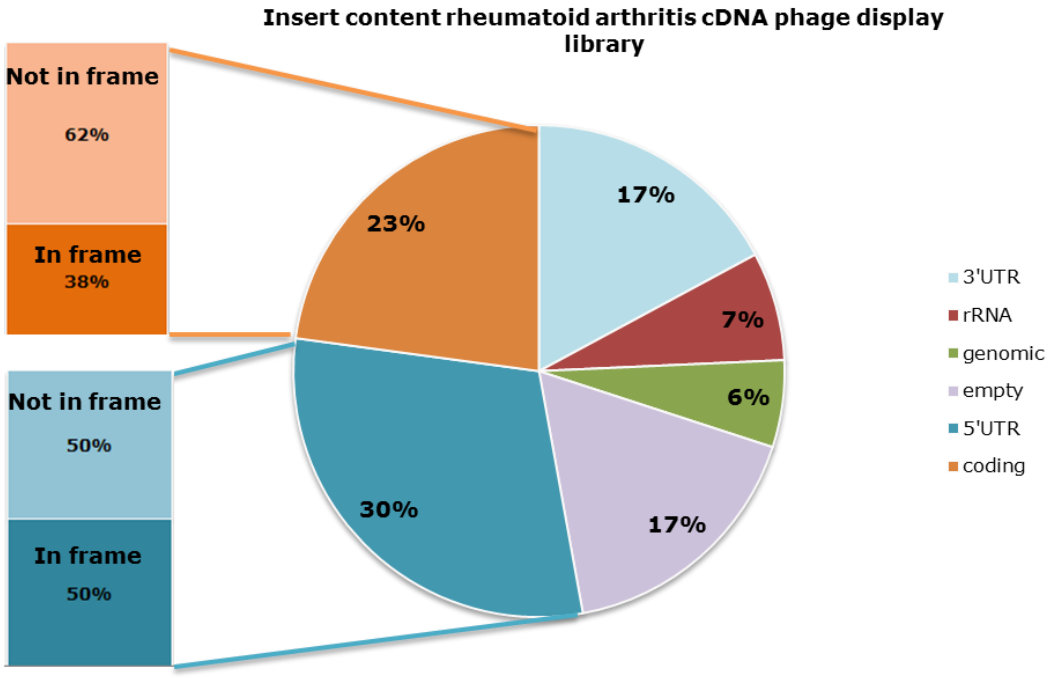

Figure 11: Identification of 70 inserts of the human RA cDNA phage display library containing a diversity of inserts corresponding to 5' or 3' untranslated regions (UTR) of expressed genes, coding region, genomic DNA (genomic) and ribosomal RNA (rRNA). Only 17% were empty vectors.

Analysis of the cDNA content provided a snapshot of the genes expressed in the synovial tissues: the most abundant type of genes were coding for ribosomal proteins but also for proteins involved in the immune response, inflammation and cartilage turnover, which can be interesting in the AS pathogenesis (**Table 9**) (**Supplement 2: Sequencing results of the inserts of the human RA cDNA phage display library**). Gene expression data of AS suggests the involvement of the immune system, extracellular or intracellular signaling pathways and bone matrix biosynthesis (42). These systems are also represented in the human RA cDNA phage display library. The extracellular protein, collagen type I alpha 1, is expressed in the human RA cDNA phage display library and is a characteristic synovial component that is involved in the bone matrix biosynthesis. Markers involved with collagen can give an indication of disease activity in AS (39, 43). Another expressed extracellular matrix protein is fibronectin 1, which is found in the synovium of chronic inflammatory joint diseases, like AS (44). More extracellular proteins in the human RA cDNA phage display library are lysyl oxidase-like 1, involved in the biosynthesis of connective tissue and cathepsin B, which can possibly play a role in the inflammation process of AS by degrading CRP (45). Next, the RA inserts also encoded proteins with a cytoplasmic localization, such as ferritin, tropomyosin and actin of which the last two are involved in the cytoskeleton functioning. Subunits of ferritin are involved in the iron storage and are upregulated in RA synovium (43). Also the expression of S100A9, a subunit of the cytoplasmic calprotectin protein complex, is interesting for AS

pathogenesis, because some studies state that calprotectin can predict radiographic progression in AS patients (9). An important protein, which is expressed in the human RA cDNA library and that has a localization on the plasma membrane is the HLA class II histocompatibility antigen gamma chain (CD74). This protein is involved in the immune response by antigen processing and a high prevalence of autoantibodies against CD74 was found in AS patients (27). Regardless if these cDNA inserts are fused in frame or not with the geneVI in our analysis, the fact that they are expressed in this human RA cDNA library, confirms that it can be used in the screening for specific AS Ab reactivity.

Table 9: Sequencing of the human RA cDNA phage display library with genes of interest for AS. Fusion types were: 5'untranslated (UTR), coding and 3'UTR. Also if the fusion of the inserts with the geneVI happened in frame or not is indicated: Y: yes; N: no; N/A: not applicable.

Phage Clone	NCBI code	cDNA identity	Fusion type	In frame
4	NM_153649.3	tropomyosin 3 (TPM3), variant 1 (TPM3)	5'UTR	Y
5	NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	5'UTR	Y
6	NM_000146.3	ferritin, light polypeptide (FTL)	5'UTR	Y
10	NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	5'UTR	N
17	NM_000088.3	collagen type I alpha 1 (COL1A1)	5'UTR	N
22	NM_212478.2	fibronectin 1 (FN1)	coding	Y
32	NM_002965.3	S100 calcium binding protein A9 (S100A9)	coding	N
33	NM_001101.3	actin, beta (ACTB)	coding	N
39	NM_001908.4	Cathepsin B (CTSB)	3'UTR	N/A
40	NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	3'UTR	N/A
42	NM_001025159.2	major histocompatibility complex, class II invariant chain (CD74)	3'UTR	N/A
43	NM_001101.3	actin, beta (ACTB)	3'UTR	N/A

The human RA cDNA phage display library is a diverse library of high quality, containing few empty pSPVI clones, which has a good distribution of the three different pSPVI-vectors and of the three RA patients. The cDNA inserts have a broad range of different sizes and are relevant in the AS pathogenesis, making this a good cDNA library to screen for AS specific antibody reactivity.

3.2.2. Antibody reactivity antigenic targets of human RA cDNA phage display library

Previously, the human RA cDNA phage display library has been screened for Ab reactivity using SAS. Successive rounds of positive selection using pooled sera from 10 AS patients and a round of negative selection, using age and gender matched HCs, allowed isolation of specific AS-related antibody targets. Identification of the SAS output resulted in 67 different Ab targets, which have a length ranging from 3 to 69 amino acids. The majority of Ab targets (92,5%) encoded novel artificial peptides, resulting from out-of-frame fusion of the cDNA insert to the M13 gene VI, while five phage clones (7,5%) encoded a known protein-coding gene fused in frame to the phage coat protein. All 67 Ab targets were screened individually for Ab reactivity in the AS plasma pool, of which 64 targets had a ratio (signal target in AS pool/signal empty phage in AS pool) above 1.5. These 64 Ab targets were further tested for Ab reactivity in the HC plasma pool. Ab targets that showed Ab reactivity in HC plasma, represented by a ratio (signal target in HC pool/ signal empty phage in HC pool) above 1.5, were excluded. From the resulting 29 Ab targets, the detected Ab reactivity's in the HC pool and AS pool were corrected for non-specific signal by subtraction of the empty phage signal. Subsequently, the difference between Ab reactivity in the HC pool and AS pool against the 29 targets was calculated. Only targets that have a subtraction of these two signals (Δ AS – HC) above 0.5 were selected, to allow further screening of 21 antibody targets that showed a higher antibody reactivity for the AS plasma pool as compared to the HC plasma pool (**Table 10**). This led to the determination of the Ab reactivity of 21 targets in individual plasma samples of the AS and HC pool, that were prior used during the SAS selection, by phage ELISA. We only tested the Ab reactivity in nine of ten individual plasma samples of the AS and HC pool, as one patient showed to be poly-reactive against the empty phages.

This resulted in a lot of heterogeneity in Ab reactivity, because each phage was reactive against only one plasma sample (**Figure 12**) (**Supplement 3: Result phage ELISA of the 21 antigenic targets**). On the other hand, the majority of patients were reactive against more than one target. Two patients did not show any reactivity against all tested targets, which is not unexpected as the used plasma samples were used as a pool during SAS selection. Further, no reactivity against any of these phage displayed peptide was found in the plasma samples of individual HC. These results illustrate Ab reactivity in plasma of AS patients.

Table 10: 21 phage clones that were used for testing the Ab reactivity in individual plasma samples. Reactivity was measured in the healthy control (HC) pool and AS pool two times each. The average of these measurements were corrected for non-specific signal. The subtraction of both corrected values (ΔAS-HC) had to be above 0.5 to be included in the individual plasma screening.

Phage clone	HC1	HC2	Average HC	HC corrected	AS1	AS2	Average AS	AS corrected	ΔAS-HC corrected
1	0.378	0.224	0.301	0.036	1076	0.797	0.937	0.723	0.687
2	0.297	0.284	0.291	0.026	0.932	1005	0.969	0.755	0.729
3	0.212	0.313	0.263	0.053	1167	1028	1098	0.926	0.874
4	0.221	0.198	0.210	-0.001	0.746	0.704	0.725	0.554	0.554
5	0.220	0.338	0.279	0.069	0.865	1108	0.987	0.815	0.746
6	0.204	0.181	0.193	-0.018	1.531	1.747	1.639	1.468	1.485
7	0.224	0.197	0.211	0.001	1.155	0.895	1.025	0.854	0.853
8	0.218	0.193	0.206	-0.005	1.266	1.224	1.245	1.074	1.078
9	0.209	0.283	0.246	0.036	0.786	0.937	0.862	0.690	0.654
10	0.252	0.258	0.255	0.045	0.894	1.048	0.971	0.800	0.755
11	0.445	0.210	0.328	0.118	0.726	0.871	0.799	0.627	0.510
12	0.225	0.200	0.213	0.016	1.091	1.096	1.094	0.940	0.924
13	0.238	0.219	0.229	0.032	1.098	1.108	1.103	0.949	0.917
14	0.265	0.211	0.238	0.042	1.504	1.344	1.424	1.270	1.229
15	0.234	0.206	0.220	0.024	1.209	1.357	1.283	1.129	1.106
16	0.245	0.225	0.235	0.039	1.049	1.113	1.081	0.927	0.889
17	0.217	0.201	0.209	0.013	1.299	1.295	1.297	1.143	1.131
18	0.184	0.232	0.208	0.012	0.676	0.767	0.722	0.568	0.556
19	0.187	0.188	0.188	-0.009	1.033	1.328	1.181	1.027	1.036
20	0.210	0.202	0.206	0.009	1.120	1.260	1.190	1.036	1.027
21	0.198	0.385	0.292	0.095	0.821	0.747	0.784	0.630	0.535

		Antibody targets																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Plasma samples	AS-RA p01									■	■						■	■					4/21	# Reactive plasma
	AS-RA p02																						0/21	
	AS-RA p03																						0/21	
	AS-RA p04					■		■				■										■	4/21	
	AS-RA p05	■	■																				2/21	
	AS-RA p06			■	■														■		■		4/21	
	AS-RA p07						■								■					■			3/21	
	AS-RA p08								■					■									2/21	
	AS-RA p09												■			■							2/21	
		1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9	1/9		
		# Reactive antibody targets																						

Figure 12: Overview of antibody reactivity of AS patients against 21 antibody targets. Horizontally the 21 different phage clones are shown and vertically the 9 different plasma samples from 9 different AS patients. The boxes indicate when there is reactivity between target and plasma.

4. Discussion and outlook

In current clinical practice, the diagnosis of AS is still very difficult and often delayed for several years. The used ASAS criteria, which support the diagnosis, are established for classification and not for diagnosis. Classification criteria are created to distinguish patients with the disease and healthy subjects in contrast to diagnostic criteria, which are made for individual patients to give them a correct diagnosis (32, 46). In the ideal situation, classification and diagnostic criteria could be the same, if sensitivity and specificity were both 100%, allowing the classification criteria to apply to every individual case (46). This is unfortunately almost never the case and this leads to misclassification of some patients, over- or underestimating the frequency of the disease (3, 46). Also in contrast to the application of classification criteria, a simple yes or no answer can generally not be achieved in a diagnostic setting (32). This makes a diagnostic approach more flexible than a classification, because negative findings will also be taken into account at the final diagnosis based on an expert's opinion (32). This is thus the gold standard to diagnose in rheumatology: the judgment of the responsible rheumatologist, who considers features of an individual patient beyond those represented in the criteria (32, 46).

As there is no specific serological test or a pathognomonic disease characteristic, rheumatologists rely often on radiological imaging. However, changes visible on radiological imaging often appear years after the disease onset of AS. This causes a considerable diagnosis delay (30). There is thus a high need for good biomarkers to make the diagnosis easier and faster. Although the pathogenesis of AS is not entirely clear, by the involvement of both genetics and environmental triggers and different underlying disease processes, it is believed to be a multifactorial disease (1, 39). Also the underlying processes of AS, like damage and inflammation, are not only specific for this disease, resulting in quantitative rather than qualitative differences in these processes between different chronic arthritis (39). It seems thus unlikely that a single marker can be found that can serve as a general diagnostic tool, covering the heterogeneity of the disease and being highly sensitive and specific. Therefore, the aim is often to develop a panel of biomarkers. In this project it was tried to find antibodies specific for AS that could serve as potential biomarkers facilitating the diagnosis of AS since the involvement of the humoral immune response in AS is gaining more attention.

First, a human AS cDNA phage display library was constructed from synovial tissue of five AS patients. However, the constructed library had a too low diversity as only 10^5 cfu could be generated after electroporation of the TG1 electrocompetent cells. Because there are between 19.000 and 20.500 (10^5) different protein coding genes in the human genome and each of these genes is not represented equally within a cDNA repertoire, a cDNA phage display library of a cDNA repertoire with a miniumum size of 10^6 should be generated (47, 48).

As our group is experienced with the cDNA phage display technique (43, 49), the reason why the diversity of this library was so low was investigated. Almost 50% of 55 clones tested clones of the human AS cDNA phage display library did not contain an insert, in contrast to the already constructed human RA cDNA phage display library, which only contained 18.5% empty clones. This

indicates that the human AS cDNA phage display library has a very low quality in comparison with the RA library.

A possible reason, that the library has such a low diversity and low quality, is the poor integrity of the RNA from which the cDNA was constructed. This could make it difficult to ligate this cDNA with different adaptors in a vector, as the cDNA synthesis could not be done properly (50). Important steps for high quality RNA are: collection and stabilization, disruption and homogenization and purification. Stabilization of RNA is very important, as RNA is a very unstable molecule and can be rapidly degraded in the presence of nearly ubiquitous RNase enzymes. Upon surgical collection, our samples were placed as soon as possible into liquid nitrogen to stabilize the RNA. Also the isolation of RNA from these samples took place in a cryostat at -20°C and was performed as fast as possible, ensuring that the tissue could not thaw. Further, the isolation of RNA was done in RNase free environment as well as possible. For example, the used solutions were treated with 0.1% diethylpyrocarbonate , a strong inhibitor of RNase. It is also possible that tissue itself is not of a good quality, causing degradation of the RNA. For example, a synovial tissue sample can contain a lot of cartilage, which hinders the isolation of sufficient amounts of high quality RNA, because cartilage has a low cell content of only 1-5% of the total mass, it contains large amounts of proteoglycans in the highly crosslinked extracellular matrix and cartilage samples of arthritis patients contains considerably enhanced levels of RNA degradation (51). Next, there could be a difference between tissues of different patients, but also between tissues deriving from the same patient, but from another pathogenic site. In our results, a difference was noted between the tissues deriving from the hip and the ones that derived from the spine. Spinal tissue obtained a higher RNA concentration than the hip tissue, however our analysis indicated that the integrity of the RNA from spinal tissue was lower in comparison to the hip tissue. Good quality total RNA should have a two clear ribosomal peaks: the 18S and 28S and a low amount of smaller-sized breakdown products. The RIN gives also an indication for the quality of the RNA, with a score from 1-10, which represents very degraded to highly intact RNA. All AS tissues had a low RIN value and a high amount of small-sized degradation products, with the RIN values of the spinal tissues being extremely low. Thus, despite the high concentration of the RNA isolated from the spinal tissue, its quality was very poor. This difference in RNA yield and integrity between spinal and hip tissue can be possible by different tissue composition. Lipid-rich tissues can deliver large yields RNA if all techniques are optimized, however lipids can complicate the RNA extraction process, because they hinder the clean separation of RNA. In contrast to lipid-rich tissue, the yield of total RNA for fibrous tissue is typically low, due to low cell density and the polynucleated nature. As it is difficult to completely disrupt all cells and to homogenize large chunks of fibrous tissue, RNA isolation of fibrous tissue can result in degraded RNA and very low yield (52).

Because of the low diversity of the human AS cDNA phage display library, we decided to continue with the previously constructed human RA cDNA phage display library to screen for AS specific antibodies. However, the construction of an AS library will still be interesting in future research, because AS samples likely contain distinct expression profiles in comparison with RA, because of their different pathologies, as there is no new bone formation in RA (16). Our lab has the unique opportunity to work with AS samples from sites of disease activity, such as our synovial biopsies

from sacroiliac or spinal joints, since biopsies of inflamed or ankylosed joints are very rarely performed, which poses a major hurdle in most studies (16). Moreover, the use of affected tissue would give the display library the whole heterogeneous antigen composition of the disease, characterized by inflammation, ossification and tissue destruction. Another advantages of screening affected tissue with the use of cDNA phage display library is that unknown or uncharacterized protein targets are also included in the antigenic diversity in contrast to antigen arrays (43).

cDNA phage display screenings are however less commonly used, as the library construction and expression can involve technical difficulties. Also as the system uses a bacterial expression system, there are no post-translational modifications beside the formation of disulfide bonds. However phage display is a powerful tool to screen for protein-protein interactions, which has proven to be a very capable method for the isolation of antibodies to defined target antigens (53). It can generate a large repertoire of cDNAs and it allows enrichments of phages with a specific cDNA insert, as it directly links the genotype and the phenotype. Also it can quickly identify interacting partners on both homogenous ligands such as monoclonal antibodies or heterogeneous ligands such as patient sera. Together these characteristics make phage display a valuable tool for the identification of immunogenic targets in autoimmune diseases (47). Our group already demonstrated that phage display library can be an unbiased, multiplexed autoantibody profiling approach for several diseases involving the formation of autoantibodies (43).

We worked with the M13 filamentous phage, which is a non-lytic phage, meaning that it will not lyse the host bacteria during phage production. This has the main advantage that the intermediate phage purification steps during phage production are greatly simplified, as a simple PEG precipitation step is sufficient to remove the phage from most contaminating cellular proteins (54). The M13 filamentous phage consists out of five different coat proteins: a major coat protein pVIII, and four minor coat proteins pIII, pVI, pVII and pIX (55). We created our phage display library by using the pVI display system, which makes C terminal fusion to the ligands possible. This enables the creation of full length cDNA, which is more hindered by N-terminal display systems, like fusion to the pIII coat protein, due to inherent stop codons present in the cDNA (47). The pVI fusion is monovalent, which enables the generation of phage particles with one fusion protein per phage particle (56). Also the use of a phagemid format, a geneVI-expressing plasmid in combination with a helper phage system, causes a monovalent display (57). Together, this allows identification of high-affinity interactions.

The construction of the human AS cDNA phage display library should be optimized before it can be used as a screening tool. To begin, the RNA quality should be better before continuing with cDNA synthesis. We now used Qiazol for the isolation of RNA, which is a lysis reagent optimized for the lysis of fatty tissue as stated by its manufacturer, Qiagen. A lysis buffer that is optimized for the lysis of fiber rich tissue could be an interesting alternative. Another factor that can be optimized is the insert:vector ratio for the ligation step. 3:1 insert:vector ratio was now used, however it could be that other ratios could be more efficient for the ligation for each different fraction. After optimization of the construction of the AS cDNA phage display library, a new serological antigen selection can be performed, screening for specific antigen-antibody reactivity against disease-related antigens in AS plasma.

A previously performed screening of AS serum antibodies against the human RA cDNA phage display library resulted in 21 different antigenic targets that were only present in AS patients. Despite the fact that this library is not derived from AS-specific disease tissue, these targets are not less valuable. It is still possible to pick up cDNA coding for antigens that are specific for AS in other libraries of rheumatic origin. For example, it can be possible that AS targets are also expressed in RA derived tissue, but not at the same frequency as in AS patients. The sequence analysis of a selection of inserts from the RA library showed that it contains a broad spectrum of different cDNA inserts, but more importantly, that it also contains inserts relevant for the AS pathogenesis. Some of these processes are involved with the immune response, inflammation and cartilage turnover. For example the insert for lysyl oxidase-like 1 was found in the RA cDNA library and is involved in the biosynthesis of connective tissue. Also the expression of S100A9, a subunit of the cytoplasmic calprotectin protein complex, is interesting for AS pathogenesis, because some studies state that calprotectin can predict radiographic progression in AS patients (9). Also the expression of the plasma membrane is the HLA class II histocompatibility antigen gamma chain (CD74) is very interesting for the pathogenesis of AS, as AS patients have a high prevalence of autoantibodies against this antigen (27). All together this makes targets found within the human RA cDNA phage display library still relevant for AS, confirming the use of this library in the screening for specific AS Ab reactivity.

The 21 different antigenic targets that were found by screening the human RA cDNA phage display library with plasma samples of AS patients indicate the presence of antibody reactivity in this disease. Although there is still no consensus about the role of the humoral immune response in AS, there are more and more indications for the presence of antibodies obtained from other studies (22, 25).

These targets look very promising as candidate diagnostic biomarkers for AS since they did not show reactivity in HC plasma and almost all the 9 AS patients showed reactivity against more than one target. However, their accuracy should be further validated in a bigger screening, as this individual screening was done against the samples that were used in the plasma pool of the SAS screening. Also two patients were not reactive against any target. Because the targets are selected after the screening of the human RA cDNA library against the serum pool, targets can be selected that are only reactive against one patients serum. If this is also a very strong interaction, it is likely to picked up by the SAS technique, regardless if the target is reactive against the other plasma samples. In a bigger screening more independent AS patients and HCs should be used, but also other rheumatic patients, like psoriatic arthritis patients and RA patients, ensuring the specificity of the targets for AS and making a distinction with the other rheumatic diseases. It is even possible to test if there is an association with a specific phenotype of AS, for example, if there is a difference in antibody reactivity against a given target in HLA-B27 positive vs HLA-B27 negative AS patients. In a later stage, synthetic peptides corresponding to the peptides displayed on the phage surface can be synthesized to confirm the binding specificity of the measured autoantibodies. Furthermore, the conversion to a peptide ELISA with our proven targets, represents a system that is more practical for clinical use compared to our phage ELISA system.

5. Conclusion

AS still needs improved diagnosis markers to shorten the delay between onset of the disease and the first treatment, preventing the irreversible destructive effects of the disease. We believe that antibodies will be a good biomarker for the diagnosis of AS. We tried to accomplish this by creating an AS cDNA phage display library, which could be screened to find an Ab biomarker specific for AS. However the cDNA library had not a high diversity, because of poor RNA quality and not optimized ligation. Therefore we worked further with the human RA cDNA phage display library. Besides their different pathologies, it is shown that the human RA cDNA phage display library contains a broad range of different insert, which are also relevant for the pathogenesis of AS. The 21 antigenic targets show Ab reactivity against plasma samples of AS patients, indicating the presence of Ab reactivity in AS and suggesting a role of the humoral immune response in the pathogenesis of AS. The value of the 21 antigenic targets as biomarkers should be further validated with screening a bigger cohort with more AS patients, other rheumatic patients and HCs. Nonetheless these 21 targets have the potential to be developed into novel biomarkers for the improved diagnosis of AS, increasing the quality of life of AS patients by decreasing irreversible damage of the axial skeleton and discomfort in the long run.

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7. Supplement

7.1. Supplement 1: Colony PCR of the inserts of the human RA cDNA phage display library

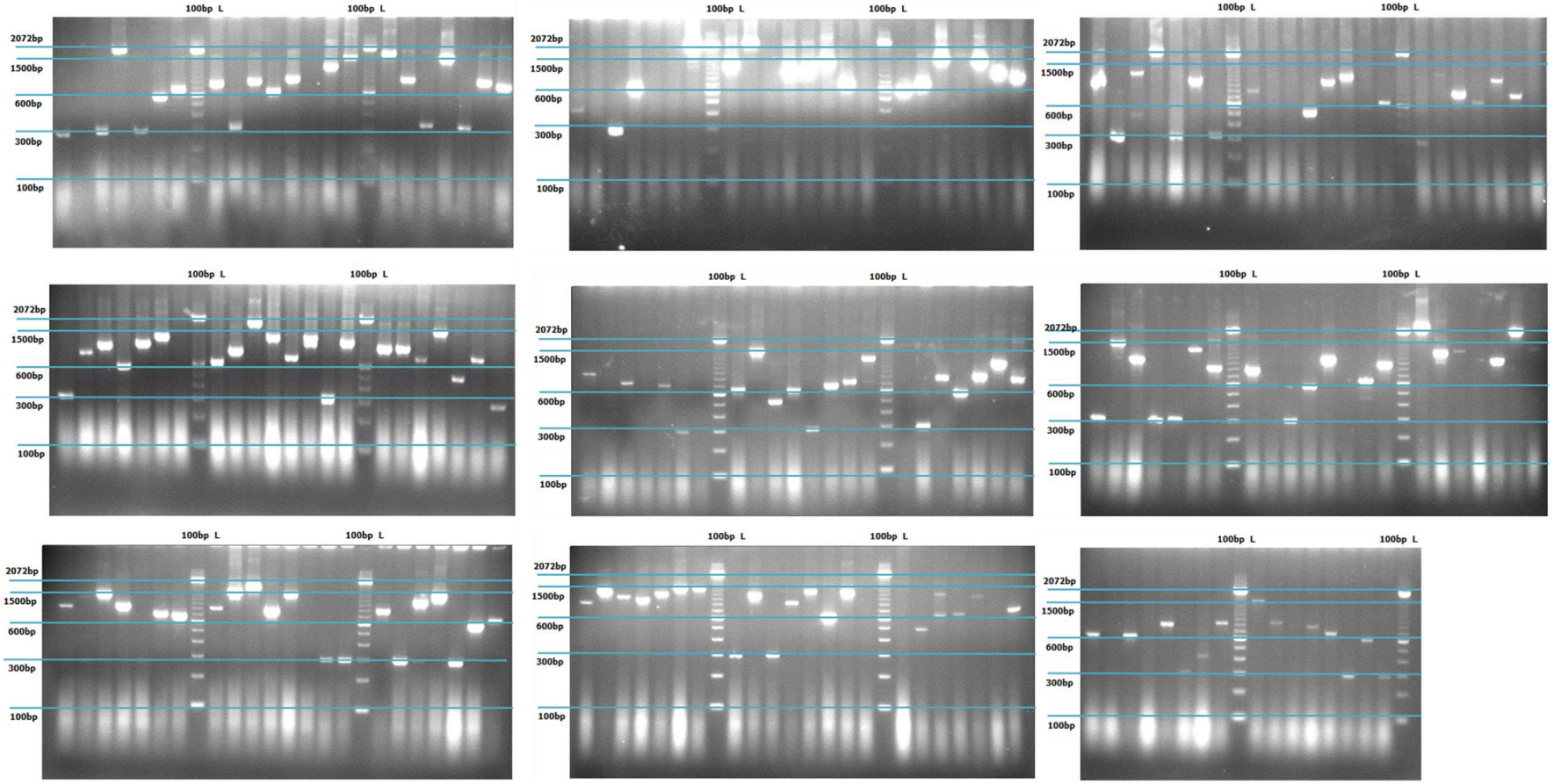

Figure 13: Colony PCR of cDNA inserts from the human rheumatoid arthritis cDNA phage display library containing a high diversity of different sized cDNA inserts. The 100base pair ladder (100bp L) was used as reference for size in bp and marked on the figure. 196 randomly selected bacterial colonies were amplified and visualized on gel resulting in 81.8% success rate. The empty vectors are visible at 300 bp on gel.

7.2. Supplement 2: Sequencing results of the inserts of the human RA cDNA phage display library

Table 11: Sequencing of the human RA cDNA phage display library . Fusion types were: 5'untranslated (UTR), coding and 3'UTR. Also if the fusion of the inserts with the geneVI happened in frame or not is indicated: Y: yes; N: no; N/A: not applicable.

Clone	NCBI code	cDNA identity	Fusion type	In frame
1	NM_000404.3	galactosidase beta 1 (GLB1)	5'UTR	Y
2	NM_000981.3	ribosomal protein L19 (RPL19)	5'UTR	Y
3	NM_002356.6	myristoylated alanine rich protein kinase C substrate (MARCKS)	5'UTR	Y
4	NM_153649.3	tropomyosin 3 (TPM3), variant 1 (TPM3)	5'UTR	Y
5	NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	5'UTR	Y
6	NM_000146.3	ferritin, light polypeptide (FTL)	5'UTR	Y
7	NM_182491.2	zinc finger, AN1-type domain 2A (ZFAND2A)	5'UTR	Y
8	NM_001017.2	ribosomal protein S13 (RPS13)	5'UTR	Y
9	NM_001024.3	ribosomal protein S21 (RPS21)	5'UTR	Y
10	NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	5'UTR	N
11	NM_005576.3	lysyl oxidase-like 1 (LOXL1)	5'UTR	N
12	NM_000992.2	ribosomal protein L29 (RPL29)	5'UTR	N
13	NM_001016.3	ribosomal protein S12 (RPS12)	5'UTR	N
14	NM_006423.2	Rab acceptor 1 (prenylated) (RABAC1)	5'UTR	N
15	NM_002933.4	ribonuclease A family member 1 (RNASE1)	5'UTR	N
16	NM_021104.1	ribosomal protein L41 (RPL41)	5'UTR	N
17	NM_000088.3	collagen type I alpha 1 (COL1A1)	5'UTR	N
18	NM_015683.1	arrestin domain containing 2 (ARRDC2)	5'UTR	N
19	NM_012423.3	ribosomal protein L13a (RPL13A)	5'UTR	N
20	NM_153741.1	dolichyl-phosphate mannosyltransferase polypeptide 3 (DPM3)	5'UTR	N
21	NM_002948.3	ribosomal protein L15 (RPL15)	5'UTR	N
22	NM_212478.2	fibronectin 1 (FN1)	coding	Y
23	NM_006600.3	nuclear distribution protein (NUDC)	coding	Y
24	NM_031369.2	heterogeneous nuclear ribonucleoprotein D (HNRNPD)	coding	Y
25	NM_000975.3	ribosomal protein L11 (RPL11)	coding	Y

26	NM_022551.2	ribosomal protein S18 (RPS18)	coding	Y
27	NM_001357.4	DEAH-box helicase 9 (DHX9)	coding	Y
28	NM_000177.4	Gelsolin (GSN)	coding	Y
29	NM_005103.4	fasciculation and elongation protein zeta 1 (FEZ1)	coding	Y
30	NM_000062.2	serpin peptidase inhibitor, clade G (SERPING1)	coding	N
31	NM_001015.4	ribosomal protein S11 (RPS11)	coding	N
32	NM_002965.3	S100 calcium binding protein A9 (S100A9)	coding	N
33	NM_001101.3	actin, beta (ACTB)	coding	N
34	NM_002775.4	HtrA serine peptidase 1 (HTRA1)	coding	N
35	NM_001015.4	ribosomal protein S11 (RPS11)	coding	N
36	NM_001303114.1	tubulin alpha 1c (TUBA1C)	coding	N
37	NM_001011.3	ribosomal protein S7 (RPS7)	coding	N
38	NM_023080.2	C8orf33 (C8orf33)	3'UTR	N/A
39	NM_001908.4	Cathepsin B (CTSB)	3'UTR	N/A
40	NM_002032.2	ferritin, heavy polypeptide 1 (FTH1)	3'UTR	N/A
41	NM_014045.3	low density lipoprotein receptor-related protein 10 (LRP10)	3'UTR	N/A
42	NM_001025159.2	major histocompatibility complex, class II invariant chain (CD74)	3'UTR	N/A
43	NM_001101.3	actin, beta (ACTB)	3'UTR	N/A
44	NM_182547.3	transmembrane p24 trafficking protein 4 (TMED4)	3'UTR	N/A
45	NM_002615.5	serpin peptidase inhibitor, clade F (SERPINF1)	3'UTR	N/A
46	NM_014604.3	(human T-cell leukemia virus type I) binding protein 3 (TAX1BP3)	3'UTR	N/A
47	NM_207012.2	adaptor related protein complex 3 mu 1 subunit (AP3M1)	3'UTR	N/A
48	NM_173179.3	solute carrier family 35 (GDP-fucose transporter), member C2 (SLC35C2)	3'UTR	N/A
49	NM_015360.4	Ski2 like RNA helicase 2 (SKIV2L2)	3'UTR	N/A
50	NR_046235.1	45S pre-ribosomal 5 (RNA45S5)	rRNA	N/A
51	NR_046235.1	45S pre-ribosomal 5 (RNA45S6)	rRNA	N/A
52	NR_046235.1	45S pre-ribosomal 5 (RNA45S7)	rRNA	N/A
53	NR_046235.1	45S pre-ribosomal 5 (RNA45S8)	rRNA	N/A
54	NR_003286.2	18S ribosomal 5 (RNA18S5)	rRNA	N/A

55	(genomic)	genomic	N/A
56	(genomic)	genomic	N/A
57	(genomic)	genomic	N/A
58	(genomic)	genomic	N/A

7.3. Supplement 3: Result phage ELISA of the 21 antigenic targets

Table 12: Result phage ELISA of the 21 phage clones. Average optical density (OD) was measured and the standard deviation (SD) and coefficient variance (CV) were calculated for both the signal of the sera and the background sera. The ratio and the subtraction out of these ODs are also calculated and the resulting average and SD of these subtractions. These are used to calculate the cutoff value. Positive samples have a subtraction value above the cutoff value.

Phage clone 1:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Sub-traction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.08	0.00	3.22	0.07	0.00	1.48	1.07	0.00			
AS-RA p02	0.08	0.01	14.56	0.08	0.00	1.86	0.99	0.00			
AS-RA p03	0.07	0.00	4.84	0.06	0.00	5.07	1.05	0.00			
AS-RA p04	0.12	0.01	7.99	0.13	0.01	7.07	0.88	-0.02			
AS-RA p05	0.89	0.07	8.02	0.10	0.00	2.15	8.99	**0.79**			
AS-RA p06	0.09	0.00	1.63	0.08	0.00	3.39	1.04	0.00			
AS-RA p07	0.09	0.00	4.31	0.09	0.01	7.57	1.02	0.00			
AS-RA p08	0.09	0.01	8.89	0.08	0.01	8.16	1.12	0.01			
AS-RA p09	0.24	0.02	6.30	0.14	0.00	1.50	1.71	0.10	**0.06**	**0.19**	**0.61**
AS-RA HC01	0.09	0.01	11.51	0.08	0.01	6.98	1.06	0.00			
AS-RA HC02	0.09	0.01	13.30	0.09	0.01	12.64	0.98	0.00			
AS-RA HC03	0.31	0.03	8.92	0.19	0.01	3.16	1.62	0.12			
AS-RA HC04	0.09	0.00	3.21	0.08	0.00	1.70	1.06	0.01			
AS-RA HC05	0.08	0.00	2.16	0.08	0.00	3.70	1.07	0.01			
AS-RA HC06	0.09	0.00	4.82	0.08	0.00	2.92	1.04	0.00			
AS-RA HC07	0.07	0.00	0.00	0.07	0.00	0.53	0.99	0.00			
AS-RA HC08	0.07	0.01	14.09	0.08	0.01	6.53	0.90	-0.01			
AS-RA HC09	0.10	0.00	1.80	0.10	0.01	11.16	1.00	0.00			

Phage clone 2:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.08	0.00	0.47	0.07	0.00	1.48	1.06	0.00			
AS-RA p02	0.07	0.00	4.78	0.08	0.00	1.86	0.97	0.00			
AS-RA p03	0.06	0.01	15.21	0.06	0.00	5.07	1.00	0.00			
AS-RA p04	0.12	0.00	2.10	0.13	0.01	7.07	0.91	-0.01			
AS-RA p05	1.36	0.03	2.23	0.10	0.00	2.15	13.83	**1.26**			
AS-RA p06	0.09	0.00	4.09	0.08	0.00	3.39	1.04	0.00			
AS-RA p07	0.09	0.01	13.53	0.09	0.01	7.57	0.97	0.00			
AS-RA p08	0.09	0.01	9.85	0.08	0.01	8.16	1.15	0.01			
AS-RA p09	0.32	0.01	3.24	0.14	0.00	1.50	2.25	0.18	**0.09**	**0.30**	**0.99**
AS-RA HC01	0.08	0.01	10.46	0.08	0.01	6.98	0.96	0.00			
AS-RA HC02	0.09	0.01	9.87	0.09	0.01	12.64	0.96	0.00			
AS-RA HC03	0.42	0.03	7.68	0.19	0.01	3.16	2.20	0.23			
AS-RA HC04	0.09	0.00	2.47	0.08	0.00	1.70	1.04	0.00			
AS-RA HC05	0.08	0.00	5.27	0.08	0.00	3.70	1.05	0.00			
AS-RA HC06	0.09	0.00	5.76	0.08	0.00	2.92	1.01	0.00			
AS-RA HC07	0.09	0.00	1.17	0.09	0.00	5.47	1.00	0.00			
AS-RA HC08	0.08	0.00	6.60	0.08	0.01	6.53	0.92	-0.01			
AS-RA HC09	0.11	0.00	4.37	0.12	0.02	19.79	0.85	-0.02			

Phage clone 3:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.06	0.00	1.82	0.07	0.00	0.05	0.89	-0.01			
AS-RA p02	0.06	0.00	4.68	0.08	0.01	0.14	0.75	-0.02			
AS-RA p03	0.06	0.00	1.25	0.07	0.01	0.12	0.82	-0.01			
AS-RA p04	0.07	0.00	1.53	0.08	0.01	0.17	0.90	-0.01			
AS-RA p05	0.09	0.01	10.81	0.09	0.01	0.16	0.99	0.00			
AS-RA p06	1.00	0.04	4.15	0.08	0.01	0.18	11.96	**0.92**			
AS-RA p07	0.09	0.00	0.41	0.10	0.02	0.20	0.90	-0.01			
AS-RA p08	0.07	0.01	8.26	0.08	0.01	0.18	0.89	-0.01			
AS-RA p09	0.09	0.01	10.05	0.10	0.00	0.01	0.92	-0.01	**0.06**	**0.19**	**0.61**
AS-RA HC01	0.07	0.00	0.48	0.08	0.00	0.04	0.89	-0.01			
AS-RA HC02	0.07	0.00	4.88	0.09	0.00	0.04	0.83	-0.02			
AS-RA HC03	0.13	0.02	17.34	0.16	0.01	0.09	0.83	-0.03			
AS-RA HC04	0.06	0.00	3.17	0.07	0.01	0.08	0.80	-0.01			
AS-RA HC05	0.07	0.00	5.40	0.07	0.01	0.11	0.89	-0.01			
AS-RA HC06	0.06	0.00	0.59	0.07	0.01	0.14	0.84	-0.01			
AS-RA HC07	0.07	0.00	3.31	0.07	0.00	0.06	1.06	0.00			
AS-RA HC08	0.07	0.00	4.10	0.08	0.01	0.10	0.85	-0.01			
AS-RA HC09	0.09	0.02	19.29	0.08	0.00	0.03	1.06	0.01			

Phage clone 4:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.05	0.00	5.19	0.07	0.00	5.40	0.83	-0.01			
AS-RA p02	0.06	0.01	10.23	0.08	0.01	13.97	0.73	-0.02			
AS-RA p03	0.06	0.00	7.89	0.07	0.01	11.74	0.84	-0.01			
AS-RA p04	0.07	0.01	16.40	0.08	0.01	17.04	0.90	-0.01			
AS-RA p05	0.08	0.01	17.62	0.09	0.01	16.44	0.96	0.00			
AS-RA p06	0.57	0.05	9.39	0.09	0.01	9.16	6.45	**0.48**			
AS-RA p07	0.07	0.01	14.67	0.08	0.00	0.44	0.83	-0.01			
AS-RA p08	0.07	0.01	11.33	0.08	0.01	18.22	0.88	-0.01			
AS-RA p09	0.09	0.00	3.43	0.10	0.00	0.71	0.93	-0.01	**0.02**	**0.12**	**0.37**
AS-RA HC01	0.07	0.00	0.97	0.08	0.00	3.82	0.88	-0.01			
AS-RA HC02	0.07	0.00	5.24	0.09	0.00	4.04	0.85	-0.01			
AS-RA HC03	0.13	0.02	15.04	0.16	0.01	9.01	0.82	-0.03			
AS-RA HC04	0.05	0.00	3.89	0.07	0.01	8.08	0.78	-0.02			
AS-RA HC05	0.06	0.00	2.86	0.07	0.01	10.58	0.84	-0.01			
AS-RA HC06	0.06	0.00	3.01	0.07	0.01	13.75	0.82	-0.01			
AS-RA HC07	0.07	0.01	9.43	0.07	0.00	5.54	0.96	0.00			
AS-RA HC08	0.07	0.00	3.07	0.08	0.01	10.01	0.85	-0.01			
AS-RA HC09	0.09	0.01	7.27	0.08	0.00	1.74	1.07	0.01			

Phage clone 5:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.05	0.00	0.66	0.07	0.00	5.40	0.81	-0.01			
AS-RA p02	0.05	0.01	14.00	0.08	0.01	13.97	0.62	-0.03			
AS-RA p03	0.05	0.00	3.93	0.07	0.01	11.74	0.78	-0.02			
AS-RA p04	0.61	0.08	13.15	0.08	0.01	17.04	7.92	**0.53**			
AS-RA p05	0.08	0.01	13.34	0.09	0.01	16.44	0.92	-0.01			
AS-RA p06	0.07	0.00	4.29	0.08	0.01	17.68	0.79	-0.02			
AS-RA p07	0.07	0.01	16.98	0.09	0.02	17.71	0.76	-0.02			
AS-RA p08	0.07	0.00	5.20	0.08	0.01	18.22	0.83	-0.01			
AS-RA p09	0.09	0.01	7.23	0.10	0.00	0.71	0.88	-0.01	**0.02**	**0.13**	**0.40**
AS-RA HC01	0.07	0.00	0.49	0.08	0.00	3.82	0.87	-0.01			
AS-RA HC02	0.08	0.00	2.35	0.09	0.00	4.04	0.86	-0.01			
AS-RA HC03	0.12	0.02	15.37	0.16	0.01	9.01	0.73	-0.04			
AS-RA HC04	0.06	0.01	9.43	0.07	0.01	8.08	0.80	-0.01			
AS-RA HC05	0.07	0.01	8.70	0.07	0.01	10.58	0.88	-0.01			
AS-RA HC06	0.06	0.00	7.76	0.07	0.01	13.75	0.82	-0.01			
AS-RA HC07	0.07	0.00	1.00	0.07	0.00	5.54	1.01	0.00			
AS-RA HC08	0.07	0.00	5.87	0.08	0.01	10.01	0.82	-0.02			
AS-RA HC09	0.08	0.00	3.75	0.08	0.00	1.74	0.93	-0.01			

Phage clone 6:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.05	0.01	13.34	0.07	0.01	15.36	0.79	-0.01			
AS-RA p02	0.06	0.01	9.26	0.09	0.00	1.50	0.61	-0.04			
AS-RA p03	0.06	0.01	12.43	0.08	0.00	5.07	0.78	-0.02			
AS-RA p04	0.07	0.00	0.98	0.08	0.00	3.43	0.87	-0.01			
AS-RA p05	0.07	0.01	15.93	0.09	0.00	3.99	0.80	-0.02			
AS-RA p06	0.06	0.00	1.72	0.10	0.00	2.98	0.65	-0.03			
AS-RA p07	1.28	0.03	2.43	0.10	0.00	1.75	12.68	**1.18**			
AS-RA p08	0.07	0.01	9.16	0.09	0.00	4.54	0.81	-0.02			
AS-RA p09	0.08	0.01	8.52	0.10	0.01	9.15	0.83	-0.02	**0.05**	**0.28**	**0.89**
AS-RA HC01	0.07	0.00	0.54	0.08	0.00	4.70	0.79	-0.02			
AS-RA HC02	0.07	0.00	2.03	0.08	0.00	2.20	0.87	-0.01			
AS-RA HC03	0.10	0.01	13.40	0.13	0.03	19.42	0.74	-0.03			
AS-RA HC04	0.06	0.01	18.04	0.07	0.00	5.98	0.86	-0.01			
AS-RA HC05	0.07	0.01	11.27	0.08	0.00	2.75	0.90	-0.01			
AS-RA HC06	0.06	0.01	10.14	0.08	0.01	8.78	0.74	-0.02			
AS-RA HC07	0.07	0.00	1.02	0.08	0.00	4.62	0.90	-0.01			
AS-RA HC08	0.07	0.00	6.99	0.09	0.01	9.16	0.74	-0.02			
AS-RA HC09	0.08	0.01	7.54	0.08	0.00	1.30	0.92	-0.01			

Phage clone 7:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.06	0.00	2.26	0.07	0.01	15.36	0.94	0.00			
AS-RA p02	0.07	0.00	0.50	0.09	0.00	1.50	0.75	-0.02			
AS-RA p03	0.07	0.00	5.78	0.08	0.00	5.07	0.88	-0.01			
AS-RA p04	0.90	0.05	5.25	0.08	0.00	3.43	10.93	**0.82**			
AS-RA p05	0.08	0.01	11.79	0.09	0.00	3.99	0.95	0.00			
AS-RA p06	0.08	0.00	2.26	0.10	0.00	2.98	0.82	-0.02			
AS-RA p07	0.09	0.00	1.23	0.10	0.00	1.75	0.86	-0.01			
AS-RA p08	0.08	0.00	3.20	0.08	0.00	4.85	0.96	0.00			
AS-RA p09	0.09	0.01	7.61	0.10	0.01	9.15	0.88	-0.01	**0.04**	**0.20**	**0.62**
AS-RA HC01	0.08	0.01	10.33	0.08	0.00	4.70	0.95	0.00			
AS-RA HC02	0.08	0.00	3.29	0.08	0.00	2.20	0.94	-0.01			
AS-RA HC03	0.10	0.01	12.98	0.13	0.03	19.42	0.78	-0.03			
AS-RA HC04	0.06	0.01	9.43	0.07	0.00	5.98	0.90	-0.01			
AS-RA HC05	0.07	0.01	9.03	0.08	0.00	2.75	0.92	-0.01			
AS-RA HC06	0.07	0.00	3.14	0.08	0.01	8.78	0.84	-0.01			
AS-RA HC07	0.08	0.01	12.69	0.08	0.00	4.62	0.98	0.00			
AS-RA HC08	0.07	0.01	8.09	0.09	0.01	9.16	0.84	-0.01			
AS-RA HC09	0.07	0.01	7.59	0.08	0.00	1.30	0.91	-0.01			

Phage clone 8:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.06	0.00	1.78	0.07	0.01	15.36	0.90	-0.01			
AS-RA p02	0.07	0.00	6.32	0.09	0.00	1.50	0.77	-0.02			
AS-RA p03	0.06	0.00	1.87	0.08	0.00	5.07	0.74	-0.02			
AS-RA p04	0.07	0.00	0.54	0.08	0.00	3.43	0.79	-0.02			
AS-RA p05	0.25	0.03	12.55	0.09	0.00	3.99	2.80	0.16			
AS-RA p06	0.07	0.01	7.24	0.10	0.00	2.98	0.77	-0.02			
AS-RA p07	0.08	0.00	0.00	0.10	0.00	1.75	0.77	-0.02			
AS-RA p08	0.62	0.08	12.93	0.08	0.00	4.85	7.67	**0.54**			
AS-RA p09	0.09	0.01	12.12	0.10	0.01	9.15	0.87	-0.01	**0.02**	**0.13**	**0.43**
AS-RA HC01	0.08	0.00	5.03	0.08	0.00	4.70	0.93	-0.01			
AS-RA HC02	0.07	0.00	3.87	0.08	0.00	2.20	0.91	-0.01			
AS-RA HC03	0.09	0.01	16.23	0.13	0.03	19.42	0.71	-0.04			
AS-RA HC04	0.07	0.00	7.04	0.08	0.00	5.51	0.85	-0.01			
AS-RA HC05	0.07	0.00	1.99	0.08	0.00	2.75	0.92	-0.01			
AS-RA HC06	0.06	0.01	12.02	0.08	0.01	8.78	0.77	-0.02			
AS-RA HC07	0.07	0.01	11.70	0.08	0.00	4.62	0.87	-0.01			
AS-RA HC08	0.07	0.00	3.63	0.09	0.01	9.16	0.77	-0.02			
AS-RA HC09	0.07	0.00	0.00	0.08	0.00	1.30	0.89	-0.01			

Phage clone 9:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	1.07	0.07	6.09	0.07	0.00	6.99	16.32	**1.01**			
AS-RA p02	0.05	0.01	15.57	0.09	0.00	5.79	0.64	-0.03			
AS-RA p03	0.06	0.00	2.57	0.07	0.00	6.15	0.80	-0.01			
AS-RA p04	0.07	0.00	1.57	0.08	0.01	8.26	0.88	-0.01			
AS-RA p05	0.07	0.00	0.48	0.09	0.02	17.93	0.83	-0.02			
AS-RA p06	0.06	0.01	13.91	0.09	0.01	12.79	0.67	-0.03			
AS-RA p07	0.07	0.01	12.27	0.10	0.00	0.69	0.65	-0.04			
AS-RA p08	0.06	0.00	0.55	0.08	0.00	4.62	0.83	-0.01			
AS-RA p09	0.08	0.00	2.83	0.10	0.00	0.36	0.76	-0.02	**0.04**	**0.25**	**0.79**
AS-RA HC01	0.06	0.00	1.14	0.08	0.00	1.74	0.76	-0.02			
AS-RA HC02	0.09	0.02	17.69	0.08	0.01	7.37	1.11	0.01			
AS-RA HC03	0.14	0.04	26.52	0.17	0.04	20.56	0.79	-0.04			
AS-RA HC04	0.06	0.01	19.83	0.08	0.01	12.69	0.79	-0.02			
AS-RA HC05	0.07	0.01	15.41	0.08	0.01	14.44	0.80	-0.02			
AS-RA HC06	0.06	0.01	13.96	0.08	0.01	17.62	0.71	-0.02			
AS-RA HC07	0.07	0.01	13.78	0.07	0.01	12.23	0.96	0.00			
AS-RA HC08	0.07	0.00	7.61	0.09	0.01	15.80	0.71	-0.03			
AS-RA HC09	0.08	0.00	0.00	0.09	0.01	9.59	0.89	-0.01			

Phage clone 10:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	1,08	0,06	5,55	0,07	0,00	6,99	16,38	**1,01**			
AS-RA p02	0,08	0,01	18,08	0,09	0,00	5,79	0,89	-0,01			
AS-RA p03	0,06	0,01	10,37	0,07	0,00	6,15	0,94	0,00			
AS-RA p04	0,07	0,00	1,52	0,08	0,01	8,26	0,91	-0,01			
AS-RA p05	0,08	0,01	12,86	0,09	0,02	17,93	0,93	-0,01			
AS-RA p06	0,07	0,01	12,95	0,09	0,01	12,79	0,78	-0,02			
AS-RA p07	0,08	0,00	5,37	0,10	0,00	0,69	0,77	-0,02			
AS-RA p08	0,08	0,00	3,27	0,08	0,00	4,62	0,99	0,00			
AS-RA p09	0,09	0,00	3,80	0,10	0,00	0,36	0,94	-0,01	**0,05**	**0,24**	**0,77**
AS-RA HC01	0,07	0,00	4,53	0,08	0,00	1,74	0,86	-0,01			
AS-RA HC02	0,07	0,00	6,83	0,08	0,00	0,90	0,92	-0,01			
AS-RA HC03	0,11	0,01	10,33	0,13	0,02	18,19	0,84	-0,02			
AS-RA HC04	0,07	0,01	12,30	0,07	0,01	17,20	0,93	0,00			
AS-RA HC05	0,08	0,01	8,36	0,08	0,01	14,44	0,91	-0,01			
AS-RA HC06	0,08	0,01	9,97	0,08	0,01	17,62	0,95	0,00			
AS-RA HC07	0,08	0,00	1,38	0,07	0,01	12,23	1,06	0,00			
AS-RA HC08	0,08	0,01	15,90	0,09	0,01	15,80	0,92	-0,01			
AS-RA HC09	0,09	0,00	2,38	0,09	0,01	9,59	1,01	0,00			

Phage clone 11:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.05	0.01	19.81	0.07	0.00	6.99	0.79	-0.01			
AS-RA p02	0.06	0.00	8.11	0.09	0.00	5.79	0.71	-0.02			
AS-RA p03	0.05	0.01	12.38	0.07	0.00	6.15	0.79	-0.01			
AS-RA p04	0.78	0.10	13.10	0.08	0.01	8.26	10.09	**0.70**			
AS-RA p05	0.08	0.01	13.77	0.09	0.02	17.93	0.87	-0.01			
AS-RA p06	0.06	0.01	10.97	0.09	0.01	12.79	0.67	-0.03			
AS-RA p07	0.07	0.00	2.02	0.10	0.00	0.69	0.69	-0.03			
AS-RA p08	0.07	0.00	3.17	0.08	0.00	4.62	0.88	-0.01			
AS-RA p09	0.08	0.01	8.37	0.10	0.00	0.36	0.86	-0.01	**0.02**	**0.17**	**0.53**
AS-RA HC01	0.08	0.00	0.94	0.08	0.00	1.74	0.92	-0.01			
AS-RA HC02	0.07	0.00	3.76	0.08	0.00	0.90	0.83	-0.01			
AS-RA HC03	0.10	0.02	19.25	0.13	0.02	18.19	0.73	-0.03			
AS-RA HC04	0.07	0.01	13.82	0.07	0.01	17.20	0.90	-0.01			
AS-RA HC05	0.07	0.01	7.29	0.08	0.01	14.44	0.81	-0.02			
AS-RA HC06	0.06	0.01	12.35	0.08	0.01	17.62	0.77	-0.02			
AS-RA HC07	0.07	0.00	5.24	0.07	0.01	12.23	1.03	0.00			
AS-RA HC08	0.07	0.01	7.77	0.09	0.01	15.80	0.74	-0.02			
AS-RA HC09	0.08	0.00	0.43	0.09	0.01	9.59	0.92	-0.01			

Phage clone 12:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.05	0.01	16.91	0.05	0.00	9.90	0.92	0.00			
AS-RA p02	0.06	0.01	12.63	0.05	0.01	9.69	1.02	0.00			
AS-RA p03	0.06	0.00	4.40	0.06	0.00	1.89	1.00	0.00			
AS-RA p04	0.07	0.00	1.07	0.06	0.00	2.28	1.06	0.00			
AS-RA p05	0.08	0.00	3.58	0.08	0.00	4.15	1.03	0.00			
AS-RA p06	0.06	0.00	2.19	0.06	0.00	0.56	1.02	0.00			
AS-RA p07	0.06	0.00	2.26	0.07	0.00	1.58	0.93	0.00			
AS-RA p08	0.06	0.00	7.58	0.06	0.00	1.79	0.95	0.00			
AS-RA p09	0.83	0.06	6.75	0.09	0.00	5.50	9.26	**0.74**	**0.04**	**0.18**	**0.57**
AS-RA HC01	0.06	0.00	5.48	0.07	0.00	1.04	0.95	0.00			
AS-RA HC02	0.06	0.00	8.53	0.06	0.00	2.21	0.91	-0.01			
AS-RA HC03	0.08	0.01	10.61	0.08	0.00	1.81	1.03	0.00			
AS-RA HC04	0.06	0.00	7.13	0.05	0.00	4.24	1.19	0.01			
AS-RA HC05	0.06	0.00	2.40	0.06	0.00	0.61	1.01	0.00			
AS-RA HC06	0.06	0.00	2.24	0.06	0.00	2.28	1.02	0.00			
AS-RA HC07	0.07	0.00	2.55	0.07	0.00	3.60	1.01	0.00			
AS-RA HC08	0.06	0.00	0.00	0.06	0.00	2.19	1.00	0.00			
AS-RA HC09	0.07	0.00	3.98	0.07	0.00	5.89	0.99	0.00			

Phage clone 13:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.05	0.01	11.22	0.05	0.00	9.90	0.95	0.00			
AS-RA p02	0.05	0.01	16.64	0.05	0.01	9.69	0.93	0.00			
AS-RA p03	0.06	0.00	1.92	0.06	0.00	1.89	0.98	0.00			
AS-RA p04	0.06	0.00	0.57	0.06	0.00	2.28	1.00	0.00			
AS-RA p05	0.08	0.00	1.34	0.08	0.00	4.15	1.03	0.00			
AS-RA p06	0.06	0.00	4.64	0.06	0.00	0.56	0.96	0.00			
AS-RA p07	0.06	0.00	0.57	0.07	0.00	1.58	0.93	-0.01			
AS-RA p08	0.35	0.06	15.69	0.06	0.00	1.79	5.97	**0.29**			
AS-RA p09	0.08	0.00	6.04	0.08	0.00	5.96	0.99	0.00	**0.02**	**0.07**	**0.22**
AS-RA HC01	0.07	0.00	3.19	0.07	0.00	1.04	0.98	0.00			
AS-RA HC02	0.06	0.00	2.86	0.06	0.00	2.21	0.96	0.00			
AS-RA HC03	0.08	0.01	7.00	0.08	0.00	1.81	0.97	0.00			
AS-RA HC04	0.06	0.00	3.03	0.05	0.00	4.24	1.17	0.01			
AS-RA HC05	0.06	0.00	4.07	0.06	0.00	0.61	1.04	0.00			
AS-RA HC06	0.06	0.00	0.56	0.06	0.00	2.28	1.01	0.00			
AS-RA HC07	0.07	0.00	2.05	0.07	0.00	3.60	1.00	0.00			
AS-RA HC08	0.07	0.00	2.69	0.06	0.00	2.19	1.02	0.00			
AS-RA HC09	0.07	0.00	5.89	0.07	0.00	5.89	1.00	0.00			

Phage clone 14:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.05	0.01	12.86	0.05	0.00	9.90	1.05	0.00			
AS-RA p02	0.19	0.02	11.60	0.05	0.01	9.69	3.45	0.13			
AS-RA p03	0.06	0.00	0.00	0.06	0.00	1.89	1.04	0.00			
AS-RA p04	0.06	0.00	1.10	0.06	0.00	2.28	1.04	0.00			
AS-RA p05	0.08	0.00	0.88	0.08	0.00	4.15	1.05	0.00			
AS-RA p06	0.07	0.00	5.12	0.06	0.00	0.56	1.09	0.01			
AS-RA p07	0.74	0.12	16.09	0.07	0.00	1.58	10.98	**0.67**			
AS-RA p08	0.06	0.00	0.57	0.06	0.00	1.79	1.04	0.00			
AS-RA p09	0.11	0.01	7.24	0.08	0.00	5.96	1.30	0.02	**0.05**	**0.16**	**0.52**
AS-RA HC01	0.08	0.00	3.67	0.07	0.00	1.04	1.13	0.01			
AS-RA HC02	0.07	0.00	4.73	0.06	0.00	2.21	1.05	0.00			
AS-RA HC03	0.09	0.01	7.27	0.08	0.00	1.81	1.12	0.01			
AS-RA HC04	0.06	0.00	1.69	0.05	0.00	4.24	1.26	0.01			
AS-RA HC05	0.06	0.00	4.04	0.06	0.00	0.61	1.05	0.00			
AS-RA HC06	0.07	0.00	5.70	0.06	0.00	2.28	1.10	0.01			
AS-RA HC07	0.07	0.00	0.52	0.07	0.00	3.60	0.99	0.00			
AS-RA HC08	0.07	0.00	5.35	0.06	0.00	2.19	1.13	0.01			
AS-RA HC09	0.07	0.00	4.43	0.07	0.00	5.89	1.00	0.00			

Phage clone 15:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.06	0.01	18.16	0.06	0.01	13.86	1.01	0.00			
AS-RA p02	0.06	0.01	17.52	0.05	0.01	12.50	1.05	0.00			
AS-RA p03	0.06	0.01	11.16	0.06	0.00	3.79	1.02	0.00			
AS-RA p04	0.07	0.00	2.69	0.06	0.00	1.14	1.06	0.00			
AS-RA p05	0.27	0.01	3.13	0.08	0.00	0.91	3.47	0.19			
AS-RA p06	0.07	0.00	5.36	0.07	0.00	1.06	0.99	0.00			
AS-RA p07	0.07	0.00	5.40	0.06	0.00	2.23	1.03	0.00			
AS-RA p08	0.06	0.00	2.86	0.06	0.00	0.57	1.00	0.00			
AS-RA p09	0.91	0.07	7.92	0.09	0.01	9.15	10.66	**0.82**	**0.06**	**0.20**	**0.64**
AS-RA HC01	0.07	0.00	5.54	0.07	0.00	5.70	1.03	0.00			
AS-RA HC02	0.07	0.00	3.10	0.06	0.00	0.55	1.07	0.00			
AS-RA HC03	0.08	0.00	1.27	0.09	0.01	12.05	0.95	0.00			
AS-RA HC04	0.06	0.00	0.00	0.06	0.00	1.84	1.04	0.00			
AS-RA HC05	0.06	0.00	2.89	0.06	0.00	2.42	1.05	0.00			
AS-RA HC06	0.07	0.00	7.39	0.06	0.00	0.00	1.12	0.01			
AS-RA HC07	0.07	0.00	1.03	0.06	0.00	3.85	1.07	0.00			
AS-RA HC08	0.06	0.00	2.21	0.06	0.00	4.99	1.00	0.00			
AS-RA HC09	0.08	0.01	6.86	0.07	0.00	0.98	1.14	0.01			

Phage clone 16:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.67	0.07	10.09	0.06	0.01	9.20	10.93	**0.61**			
AS-RA p02	0.06	0.00	7.64	0.05	0.01	12.50	1.03	0.00			
AS-RA p03	0.06	0.00	2.55	0.06	0.00	3.79	0.99	0.00			
AS-RA p04	0.06	0.00	0.00	0.06	0.00	1.14	1.02	0.00			
AS-RA p05	0.14	0.01	10.07	0.08	0.00	1.36	1.80	0.06			
AS-RA p06	0.07	0.00	1.60	0.07	0.00	1.06	1.00	0.00			
AS-RA p07	0.06	0.00	5.15	0.06	0.00	2.23	0.97	0.00			
AS-RA p08	0.07	0.00	4.73	0.06	0.00	0.57	1.09	0.01			
AS-RA p09	0.11	0.01	12.12	0.08	0.00	3.47	1.40	0.03	**0.04**	**0.14**	**0.47**
AS-RA HC01	0.07	0.00	4.66	0.07	0.00	5.70	1.00	0.00			
AS-RA HC02	0.08	0.00	5.44	0.06	0.00	0.55	1.21	0.01			
AS-RA HC03	0.08	0.00	3.18	0.09	0.01	12.05	0.88	-0.01			
AS-RA HC04	0.06	0.01	8.80	0.06	0.00	1.84	1.04	0.00			
AS-RA HC05	0.06	0.00	4.75	0.06	0.00	2.42	1.02	0.00			
AS-RA HC06	0.07	0.01	8.70	0.06	0.00	0.00	1.08	0.01			
AS-RA HC07	0.07	0.00	5.66	0.06	0.00	3.85	1.07	0.00			
AS-RA HC08	0.07	0.00	5.91	0.06	0.00	4.99	1.03	0.00			
AS-RA HC09	0.07	0.00	6.87	0.07	0.00	0.98	0.99	0.00			

Phage clone 17:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	1.00	0.07	6.86	0.06	0.00	3.60	17.03	**0.95**			
AS-RA p02	0.06	0.01	10.23	0.05	0.01	12.50	1.09	0.01			
AS-RA p03	0.06	0.00	0.00	0.06	0.00	3.79	1.06	0.00			
AS-RA p04	0.07	0.00	4.19	0.06	0.00	1.14	1.09	0.01			
AS-RA p05	0.08	0.00	0.00	0.08	0.00	1.36	1.01	0.00			
AS-RA p06	0.07	0.00	3.10	0.07	0.00	1.06	1.03	0.00			
AS-RA p07	0.07	0.00	2.71	0.06	0.00	2.23	1.03	0.00			
AS-RA p08	0.07	0.01	13.59	0.06	0.00	0.57	1.14	0.01			
AS-RA p09	0.09	0.01	14.76	0.09	0.00	5.30	1.05	0.00	**0.06**	**0.22**	**0.72**
AS-RA HC01	0.07	0.00	5.74	0.07	0.00	5.70	0.99	0.00			
AS-RA HC02	0.07	0.01	12.77	0.07	0.01	9.77	1.05	0.00			
AS-RA HC03	0.10	0.01	14.48	0.09	0.01	12.05	1.08	0.01			
AS-RA HC04	0.06	0.00	4.21	0.06	0.00	1.84	1.02	0.00			
AS-RA HC05	0.06	0.00	3.51	0.06	0.00	2.42	1.03	0.00			
AS-RA HC06	0.07	0.00	6.53	0.06	0.00	0.00	1.08	0.01			
AS-RA HC07	0.07	0.00	0.00	0.06	0.00	3.85	1.06	0.00			
AS-RA HC08	0.07	0.00	6.15	0.06	0.00	4.99	1.08	0.01			
AS-RA HC09	0.08	0.01	7.00	0.07	0.00	0.98	1.04	0.00			

Phage clone 18:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.06	0.00	0.55	0.06	0.01	8.52	1.03	0.00			
AS-RA p02	0.06	0.01	9.27	0.05	0.01	17.34	1.15	0.01			
AS-RA p03	0.06	0.00	8.92	0.05	0.01	18.86	1.06	0.00			
AS-RA p04	0.07	0.00	1.02	0.06	0.01	12.09	1.19	0.01			
AS-RA p05	0.08	0.00	6.56	0.07	0.01	9.93	1.01	0.00			
AS-RA p06	0.54	0.05	9.42	0.06	0.01	10.71	8.61	**0.48**			
AS-RA p07	0.06	0.00	0.55	0.06	0.00	3.31	1.00	0.00			
AS-RA p08	0.06	0.01	9.98	0.06	0.00	3.57	1.01	0.00			
AS-RA p09	0.08	0.00	2.64	0.08	0.00	1.30	0.98	0.00	**0.03**	**0.11**	**0.36**
AS-RA HC01	0.06	0.01	13.17	0.07	0.00	5.96	0.95	0.00			
AS-RA HC02	0.06	0.01	18.29	0.06	0.00	5.84	0.96	0.00			
AS-RA HC03	0.09	0.00	3.04	0.09	0.00	2.32	1.02	0.00			
AS-RA HC04	0.06	0.00	3.60	0.06	0.00	6.04	1.01	0.00			
AS-RA HC05	0.06	0.00	5.84	0.06	0.00	2.75	0.94	0.00			
AS-RA HC06	0.06	0.00	1.70	0.07	0.00	0.00	0.93	0.00			
AS-RA HC07	0.07	0.01	7.44	0.07	0.00	1.00	1.01	0.00			
AS-RA HC08	0.06	0.00	7.67	0.07	0.00	1.05	0.96	0.00			
AS-RA HC09	0.07	0.00	4.40	0.07	0.00	0.00	0.98	0.00			

Phage clone 19:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.06	0.00	6.51	0.06	0.00	1.20	1.01	0.00			
AS-RA p02	0.10	0.00	1.44	0.05	0.01	17.34	1.85	0.05			
AS-RA p03	0.06	0.00	4.48	0.05	0.01	18.86	1.05	0.00			
AS-RA p04	0.06	0.00	1.70	0.06	0.01	12.09	1.06	0.00			
AS-RA p05	0.09	0.00	0.41	0.07	0.01	9.93	1.14	0.01			
AS-RA p06	0.06	0.00	1.10	0.06	0.01	10.71	1.03	0.00			
AS-RA p07	1.00	0.09	8.68	0.06	0.00	3.31	15.65	**0.94**			
AS-RA p08	0.06	0.00	6.45	0.06	0.00	3.57	1.01	0.00			
AS-RA p09	0.08	0.00	3.16	0.08	0.00	1.30	0.96	0.00	**0.06**	**0.22**	**0.72**
AS-RA HC01	0.08	0.00	6.60	0.07	0.00	5.96	1.15	0.01			
AS-RA HC02	0.06	0.01	13.20	0.06	0.00	5.84	0.93	0.00			
AS-RA HC03	0.09	0.00	4.43	0.09	0.00	2.32	0.96	0.00			
AS-RA HC04	0.06	0.00	4.07	0.06	0.00	6.04	1.04	0.00			
AS-RA HC05	0.06	0.00	1.66	0.06	0.00	2.75	0.99	0.00			
AS-RA HC06	0.07	0.01	10.96	0.07	0.00	0.00	1.01	0.00			
AS-RA HC07	0.07	0.00	0.00	0.07	0.00	1.00	1.01	0.00			
AS-RA HC08	0.07	0.00	4.13	0.07	0.00	1.05	1.01	0.00			
AS-RA HC09	0.07	0.00	6.64	0.07	0.00	0.00	1.01	0.00			

Phage clone 20:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.06	0.00	5.66	0.06	0.00	0.60	0.96	0.00			
AS-RA p02	0.05	0.01	10.38	0.05	0.01	17.34	1.03	0.00			
AS-RA p03	0.05	0.00	6.73	0.05	0.01	18.86	1.00	0.00			
AS-RA p04	0.06	0.00	1.12	0.06	0.01	12.09	1.08	0.00			
AS-RA p05	0.08	0.00	0.46	0.07	0.01	9.93	1.02	0.00			
AS-RA p06	0.86	0.06	6.65	0.06	0.01	10.71	13.64	**0.79**			
AS-RA p07	0.06	0.00	2.24	0.06	0.00	3.31	0.98	0.00			
AS-RA p08	0.09	0.00	4.33	0.06	0.00	3.57	1.51	0.03			
AS-RA p09	0.08	0.00	0.45	0.08	0.00	1.30	0.96	0.00	**0.04**	**0.19**	**0.61**
AS-RA HC01	0.06	0.00	7.73	0.07	0.00	5.96	0.98	0.00			
AS-RA HC02	0.06	0.00	4.79	0.06	0.00	5.84	0.98	0.00			
AS-RA HC03	0.09	0.00	5.69	0.09	0.00	2.32	0.95	0.00			
AS-RA HC04	0.06	0.00	1.82	0.06	0.00	6.04	1.00	0.00			
AS-RA HC05	0.06	0.00	3.60	0.06	0.00	2.75	0.92	-0.01			
AS-RA HC06	0.06	0.00	4.01	0.07	0.00	0.00	0.92	-0.01			
AS-RA HC07	0.07	0.00	2.53	0.07	0.00	1.00	0.99	0.00			
AS-RA HC08	0.06	0.00	4.45	0.07	0.00	1.05	0.94	0.00			
AS-RA HC09	0.07	0.01	7.86	0.07	0.00	0.00	0.97	0.00			

Phage clone 21:

	Reactivity			Background							
Patient	**OD**	**SD**	**CV**	**OD**	**SD**	**CV**	**Ratio**	**Subtraction**	**average**	**SD**	**cutoff (3xSD)**
AS-RA p01	0.26	0.02	7.06	0.07	0.01	8.66	3.47	0.18			
AS-RA p02	0.10	0.00	4.63	0.07	0.00	4.34	1.35	0.03			
AS-RA p03	0.06	0.01	10.70	0.06	0.00	4.49	0.94	0.00			
AS-RA p04	0.44	0.00	0.00	0.07	0.00	3.03	6.27	**0.37**			
AS-RA p05	0.13	0.00	1.33	0.08	0.00	1.37	1.72	0.06			
AS-RA p06	0.15	0.01	3.82	0.06	0.00	2.96	2.48	0.09			
AS-RA p07	0.08	0.00	0.00	0.06	0.00	5.48	1.19	0.01			
AS-RA p08	0.08	0.00	3.94	0.06	0.00	3.60	1.37	0.02			
AS-RA p09	0.09	0.00	2.63	0.09	0.01	6.62	1.04	0.00	**0.05**	**0.09**	**0.33**
AS-RA HC01	0.06	0.00	5.61	0.07	0.00	0.54	0.97	0.00			
AS-RA HC02	0.15	0.01	4.39	0.07	0.00	3.65	2.14	0.08			
AS-RA HC03	0.09	0.00	3.76	0.09	0.01	13.83	1.02	0.00			
AS-RA HC04	0.07	0.00	2.48	0.07	0.00	2.67	1.08	0.01			
AS-RA HC05	0.07	0.01	7.60	0.06	0.00	2.55	1.26	0.01			
AS-RA HC06	0.10	0.01	8.61	0.07	0.00	2.14	1.49	0.03			
AS-RA HC07	0.07	0.00	1.44	0.07	0.00	1.06	1.10	0.01			
AS-RA HC08	0.06	0.00	0.56	0.07	0.00	1.60	0.95	0.00			
AS-RA HC09	0.07	0.00	2.36	0.07	0.00	7.12	1.08	0.01			

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
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