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ORIGINAL RESEARCH

Fibroblast-like synoviocyte targeting antibodies are associated with failure to reach early and sustained remission or low disease activity after first-line therapy in rheumatoid arthritis

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ABSTRACT

Objective To discover antibody biomarkers that can predict a lack of response to first-line therapy in rheumatoid arthritis (RA) patients.

Methods Two RA cDNA phage display libraries were screened for novel antibodies in baseline RA sera from the Care in early RA (CareRA) trial, differentiating between patients who did or did not reach remission after first-line therapy (n=20 each). Antibody reactivity to identified University Hasselt (UH)-RA antigens was validated in baseline samples from 136 additional CareRA participants. The novel antibodies' potential to predict failure to reach remission or low disease activity (LDA), according to the Disease Activity Score 28-joint C-reactive protein/erythrocyte sedimentation rate (DAS28CRP/ESR) and Clinical/Simplified Disease Activity Index (CDAI/SDAI), was studied by multivariate analyses. The presence of the antibody targets in RA synovial tissue and the fibroblast-like synoviocyte (FLS) cell line SW982 was determined by immunofluorescence.

Results We identified antibodies to 41 novel antigens. Antibodies against any of three antigens, UH-RA.305/318/329, discriminated between RA patients not reaching week (w)8 DAS28CRP remission and those that did (36% vs 13%,p=0.0031). In all patients, anti-UH-RA.305/318/329 antibody reactivity was associated with failure to reach week 8 DAS28CRP and DAS28ESR remission (OR 3.63,p=0.0031; OR 2.92,p=0.016; respectively), SDAI/CDAI sustained remission (OR 5.59,p=0.039 for both) and DAS28CRP and DAS28ESR sustained LDA (OR 3.7,p=0.009; OR 2.76,p=0.042; respectively). In rheumatoid factor/anti-citrullinated protein antibody (RF/ACPA) seronegative patients, these antibodies were strongly associated with failure to achieve week 8 DAS28CRP remission (OR 17.3,p=0.0029). Anti-UH-RA.305/329 antibodies were shown to target FLS in RA synovial tissue and SW982 cells.

Conclusion We identified three antibody biomarkers that are associated with failure to achieve remission/LDA after first-line RA therapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ About 30% of rheumatoid arthritis (RA) patients do not respond to first-line treatment with classical synthetic disease-modifying antirheumatic drugs (csDMARD), such as methotrexate (MTX) and short-term glucocorticoids. As these non-responders have to switch to biological (b)DMARD and experience progression and decreased function, there is an urgent need to identify markers that can predict response to first-line treatment.

WHAT THIS STUDY ADDS

⇒ This study identified three novel antibody biomarkers that are associated with failure to achieve remission/low disease activity (LDA) after first-line RA therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ In the long term, the novel antibody biomarkers can be a novel tool to stratify patients in responders and non-responders to RA first-line therapy and predict their response before this therapy is initiated. This strategy will allow immediate administration of the most appropriate drug to individual patients which could in some cases require accelerated access to b/targeted synthetic DMARDs.

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that affects multiple synovial joints which, if left untreated, leads to destruction of cartilage and the underlying bone. In the last two decades, a paradigm shift in RA disease management has been highly successful in driving back disease activity, resulting in reduced damage and disability for an increasing number of patients.[1]

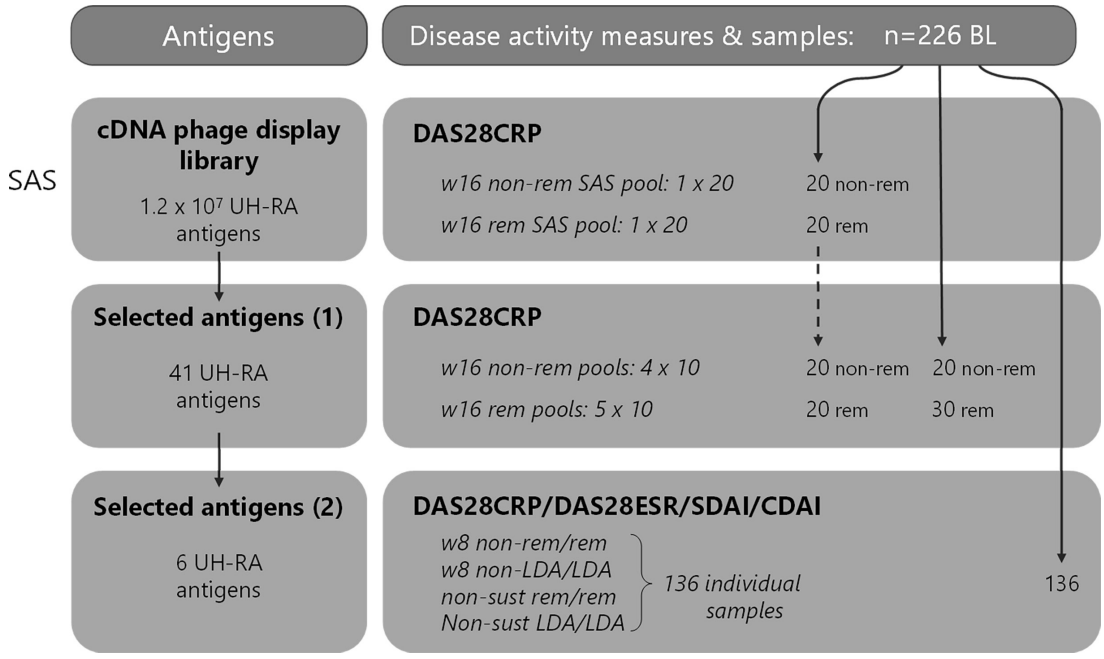

Figure 1 Flow chart of the antigens, RA baseline serum samples and disease activity measures used during SAS and subsequent determination of antibody reactivity in pooled and individual samples. Two human RA synovial cDNA phage display libraries containing 1.2×10^7 recombinant clones were used to screen for novel antibodies in pooled baseline serum from week 16 DAS28CRP non-rem patients (n=20). During SAS, baseline samples from 20 additional week 16 DAS28CRP rem patients were used for counterselection. Next, antibody reactivity against 41 UH-RA antigens identified during SAS was determined in four week 16 DAS28CRP non-rem serum pools (each consisting of 10 baseline samples, with in total 20 samples from non-rem SAS pool, and 20 additional non-rem samples), and 5 week 16 DAS28CRP rem serum pools (each consisting of 10 baseline samples, with in total 20 samples from rem SAS pool, and 30 additional rem samples). Finally, antibody reactivity against 6 selected UH-RA antigens was determined in individual baseline serum samples from 136 RA patients (new samples; not included in the SAS pool or the plasma pool). Dotted arrow indicates patients included in the SAS pool. Regular arrows indicate new patients who are not included elsewhere. Ab, antibody; BL, baseline; CDAI, Clinical Disease Activity Index; DAS28CRP, Disease Activity Score in 28 joints with C-reactive protein; DAS28ESR, DAS28 with erythrocyte sedimentation rate; LDA, patient reaching low disease activity; non-rem, patient not reaching disease remission; rem, patient reaching disease remission; RA, rheumatoid arthritis; SAS, serological antigen selection; SDAI, Simplified Disease Activity Index; sust, sustained; UH, University Hasselt; w, week.

In its 2022 update of the recommendations for the management of RA, the European Alliance of Associations for Rheumatology (EULAR) endorsed the use of classical synthetic disease-modifying antirheumatic drugs (csDMARDs), such as methotrexate (MTX), in combination with glucocorticoids (GCs) bridging as a first-line treatment.[2] The Care in early RA (CareRA) trial has shown that such a combination therapy is effective at inducing Disease Activity Score based on 28 joints with C-reactive protein (DAS28CRP) remission in about 70% of patients after 2 years and was found to be well tolerated and cost effective.[3–6] This strategy is also effective in the long term as 56% of patients did not have to intensify their DMARD treatment after 5 years.[7] However, in case of insufficient response to this first-line treatment and in the presence of poor prognostic factors, the EULAR recommendations advise escalation to biological (b) DMARDs, which are directed against cytokine signalling, B/T lymphocytes or to targeted synthetic (ts)DMARDs, which target Janus kinases.[2]

If the applied therapy is not successful in effectively suppressing inflammation, patients experience a prolonged period of high disease activity, which leads to decreased functional capacity and progression of structural joint damage.[8 9] Therefore, there is an urgent need for novel biomarkers that allow immediate administration of the most appropriate class of drugs to individual patients,[2] which could require accelerated access to b/tsDMARDs in patients who do not respond to first-line treatments. Therefore, we aimed to identify a novel tool to stratify patients into probable responders and non-responders prior to the initiation of intensive first-line therapy. To this end, we conducted an unbiased screening for novel antibody biomarkers present at baseline, capable of identifying patients who are highly likely to fail to achieve remission or low disease activity (LDA) after first-line combination therapy.

PATIENTS AND METHODS

Patients and controls

This study included data and sera from 226 patients with early RA randomly selected from the CareRA trial, recruited at 13 rheumatology centres in Belgium, and 86

Table 1 Sequence, origin and homology of 6 novel UH-RA antigens

Name UH-RA antigen	Antigen sequence*	Size (aa)†	cDNA identity (NCBI/ Ensembl accession nr.)	Fusion location ‡	In frame §	Homologous synovial proteins (Uniprot accession nr.) ¶
UH-RA.305	AAAP(V)LGYEE	10	WW domain binding protein 1 like, *WBP1L* (NM_001083913.2)	mRNA, 3'UTR	N/A	► 8/11 (73%) cardiolipin synthase, *CRLS1* (Q9UJA2) ► 6/6 (100%) superoxide dismutase, *SOD2* (P04179) ► 8/17 (47%) zinc finger protein with KRAB and SCAN domains 2, *ZKSCAN2* (Q63HK3)
UH-RA.308	(M)AGGTESRDEDKT	13	PTTG1 interacting protein, *PTTG1IP* (NM_004339.4)	mRNA, coding	No	► 8/11 (73%) microspherule protein 1, *MCRS1* (Q96EZ8) ► 7/8 (88%) interleukin-15 receptor subunit alpha, *IL15RA* (Q13261) ► 7/7 (100%) potassium/ sodium hyperpolarisation-activated cyclic nucleotide-gated channel 4, *HCN4* (Q9Y3Q4)
UH-RA.314	(S)VWGF	5	DAB adaptor protein 2, *DAB2* (NC_000005.10)	RNA, intron	N/A	► 5/5 (100%) integrator complex subunit 5, *INTS5* (Q6P9B9) ► 5/5 (100%) interleukin-27 receptor subunit alpha, *IL27RA* (Q6UWB1) ► 4/5 (80%) contactin-associated protein-like 4, *CNTNAP4* (Q9C0A0)
UH-RA.318	(V)QDSGQSPAWPASLSSSASSLTVQGPGPSLF	31	Immunoglobulin lambda variable 1–47 (ENST00000390294.2)	mRNA, 5'UTR	No	► 14/26 (54%) dynein heavy chain domain-containing protein 1, *DNHD1* (Q96M86) ► 12/17 (71%) adenomatous polyposis coli protein 2, *APC2* (O95996) ► 9/12 (75%) FH2 domain-containing protein 1, *FHDC1* (Q9C0D6)

Continued

Table 1 Continued

Name UH-RA antigen	Antigen sequence*	Size (aa)†	cDNA identity (NCBI/ Ensembl accession nr.)	Fusion location ‡	In frame §	Homologous synovial proteins (Uniprot accession nr.) ¶
UH-RA.329	QKIRGRI(D)VE	10	lysophosphatidylcholine acyltransferase 4, *LPCAT4* (NM_153613.3)	mRNA, coding	No	► 7/12 (58%) FYVE, RhoGEF and PH domain-containing protein 1, *FGD1* (P98174) ► 7/10 (70%) ATP-binding cassette sub-family D member 1, *ABCD1* (P33897) ► 6/10 (60%) ketohexokinase isoform b, KHK (P50053)
UH-RA.339	(R)LPHIPTAEGQEDGCPSSSPLCLLQQDWTFCDRRGSW	37	Gelsolin, *GSN* (NM_000177.5)	mRNA, coding	No	► 16/34 (47%) low-density lipoprotein receptor-related protein 4, *LRP4* (O75096) ► 14/25 (56%) nuclear receptor corepressor 2, *NCOR2* (Q9Y618) ► 8/14 (57%) protein phosphatase 1 regulatory subunit 3F, *PPP1R3F* (Q6ZSY5)

*Sequence of the antigen as expressed on the phage surface. aa before the bracketed aa come from translation of the cDNA cloning adaptor[14 15], while aa behind the bracketed aa originate from the translated cDNA insert. For the antigens that only express a short aa sequence originating from the cDNA insert (UH-RA.305, UH-RA.314 and UH-RA.329), the antigen sequence was determined by epitope mapping using competition ELISA.
†Size of the antigen is expressed as the number of aa.
‡The fusion location indicates the region in the RNA where the cDNA was fused to M13 gene VI.
§Type of fusion of the cDNA coding region with M13 gene VI. 'No' indicates the cDNA coding region is not in frame with M13 gene VI, resulting in out-of-frame protein expression of the cDNA insert. 'N/A' indicates the cDNA fusion occurred in a non-coding region.
¶Human synovial proteins with amino acid homology to antigen sequence, with amount and percentage of identical amino acids indicated. Top 3 hits using *RefSeq Select proteins* database on NCBI using the blastp algorithm, which are sorted by E value, and which are expressed in human synovial lymphoid, myeloid or fibroid cells according to online database from Lewis *et al*.[16]
aa, amino acid; cDNA, complementary DNA; mRNA, messenger RNA; N/A, not applicable; NCBI, National Center for Biotechnology Information; nr, number; RA, rheumatoid arthritis; RA, rheumatoid arthritis; UH, University Hasselt; 3'UTR, 3' untranslated region; 5'UTR, 5' untranslated region.

Table 2 Baseline anti-UH-RA antibody reactivity according to DAS28CRP remission at week 8

UH-RA antigen	BL Ab reactivity w8 non-rem*	BL Ab reactivity w8 rem*	LR+	P value
UH-RA.305	7/45 (15.5%)	4/91 (4.4%)	3.52	**0.033**
UH-RA.329	4/45 (8.9%)	4/91 (4.4%)	2.02	0.248
UH-RA.318	6/45 (13.3%)	7/91 (7.7%)	1.72	0.225
UH-RA.314	1/45 (2.2%)	1/91 (1.1%)	2.0	0.554
UH-RA.339	3/41 (7.32%)	6/84 (7.14%)	1.03	0.616
UH-RA.308	0/45 (0%)	2/91 (2.2%)	N/A	0.758
Panel of 3	16/45 (35.6%)	12/91 (13.2%)	2.7	**0.003**

Bold values denotes stastical significance at the p<0.05 level.
*Number and percentage of anti-UH-RA positive baseline samples from patients who did (rem) or did not (non-rem) reach DAS28CRP remission at week 8. The panel of 3 antibodies indicates antibody reactivity against at least one of the antigens namely UH-RA.305, UH-RA.318 or UH-RA.329.
Ab, antibody; BL, baseline; DAS28CRP, Disease Activity Score in 28 joints with C-reactive protein; LR+, positive likelihood ratio; N/A, not available; non-rem, patient not reaching disease remission; RA, rheumatoid arthritis; rem, patient reaching disease remission; UH, University Hasselt; W, week.

age-matched and sex-matched healthy controls (HCs), recruited at Hasselt University (Hasselt, Belgium). Details on demographics, clinical parameters, eligibility criteria, study period, treatments used and responses of the entire CareRA study were published previously.[4–6] In brief, in the CareRA trial, patients were included with a recent RA diagnosis (≤1 year) according to the American College of Rheumatology 1987 revised criteria. These patients were DMARD and GC treatment-naïve, having no contraindications for intensive GC treatment. Prior to treatment initiation, patients were stratified into a high-risk and a low-risk group, based on the presence of erosions, rheumatoid factor (RF) and/or anti-citrullinated protein antibody (ACPA), and baseline DAS28CRP.[5] High-risk patients received Combination therapy for early RA (COBRA) Classic (MTX/sulfasalazine/step-down GC), COBRA Slim (MTX/step-down GC) or COBRA Avant-Garde (MTX/leflunomide/step-down GC) and low-risk patients, received COBRA Slim/MTX monotherapy known as Tight step-up.[6]

Disease activity measures included remission and LDA according to the DAS28CRP, DAS28 with erythrocyte sedimentation rate (DAS28ESR), Simplified Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI) criteria at different time points after therapy initiation (weeks 8, 16, 28, 40, 52, 65, 78, 91 and 104). In addition, sustained (sust) remission (rem) and sust LDA over the first year were investigated, indicating remission or LDA at weeks 8, 16, 28, 40 and 52. Patients were considered non-sust rem or non-sust LDA when they failed to respectively achieve remission or LDA for at least one of these time points. The cut-offs used for classification of rem or failure to reach rem (non-rem) and LDA or failure to reach LDA (non-LDA) for the different disease activity measures are shown in online supplemental table 1.[10] Demographic and clinical parameters' details at baseline and week 16 of the various groups of patients in this study are shown in online supplemental table 2.

Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Screening for novel antibodies using serological antigen selection

Serological antigen selection (SAS) is a screening procedure that uses cDNA phage display to identify novel antibodies and their antigenic targets.[11–13] Two RA synovial cDNA phage display libraries expressing antigens from one RA hip and three RA knee synovia were used. These synovia were obtained from patients who underwent hip/knee replacement surgeries due to RA. Construction of these cDNA phage display libraries and validation of their quality, diversity and content was previously described.[14 15] The libraries have been shown to express genes and sequences encoding synovial antigens or candidate RA antigens.[14 15] To specifically isolate novel antibodies associated with failure to reach early disease remission, these phage particles were used in rounds of positive selection to isolate antigen-antibody complexes in pooled baseline sera of week 16 DAS28CRP non-rem RA patients (n=20). The patients in this non-rem SAS pool showed moderate (3.2≤DAS28CRP≤5.1) or high (DAS28CRP>5.1) disease activity at baseline, did not reach DAS28CRP remission (DAS28CRP<2.6) nor LDA (2.6≤DAS28CRP<3.2) at week 16 and showed the smallest improvement in DAS28CRP over the first 16 weeks among our study population. Negative selection was done to remove irrelevant antibody-antigen complexes using pooled baseline sera of week 16 DAS28CRP rem RA patients (n=20) (figure 1). These patients showed high DAS28CRP disease activity at baseline, reached DAS28CRP remission (DAS28CRP≥2.6) at weeks 16 and 52 and showed the

Table 3 Baseline anti-UH-RA.305/318/329 antibody reactivity according to disease remission at week 8

Disease activity measure	BL panel reactivity W8 non-rem*	BL panel reactivity W8 rem*	Univariate model†		Multivariate model‡		Covariate§	
			OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
All RA (n=135-136)¶								
W8 DAS28CRP	16/45 (36%)	12/91 (13%)	**3.63 (1.54 to 8.76)**	**0.0031**	**3.63 (1.54 to 8.76)**	**0.0031**		
W8DAS28ESR	18/57 (32%)	10/78 (13%)	**3.13 (1.34 to 7.71)**	**0.0082**	**2.92 (1.22 to 7.31)**	**0.016**	Age: 1.04 (1.0 to 1.07)	**0.0087**
W8 SDAI	21/87 (24%)	7/49 (14%)	1.91 (0.78 to 5.2)	0.163	1.61 (0.54 to 4.74)	0.384	Treatment type: 1. Avant garde-classic : 4.81 (1.65 to 13.98)	**0.0028**
							2. Slim HR-classic : 3.63 (1.27 to 10.37)	**0.0135**
							3. Tight step up-slim LR 6.38 (1.05 to 38.9)	**0.031**
W8 CDAI	21/88 (24%)	7/48 (15%)	1.84 (0.75 to 5.0)	0.191	1.73 (0.59 to 5.1)	0.323	Treatment type: 1. Avant garde-classic: 5.39 (1.81 to 1461)	**0.0016**
							2. Slim HR-classic 4.12 (1.29 to 10.51)	**0.0075**
							Seronegativity 4.45 (1.1 to 23.2)	**0.033**
RF/ACPA seronegative RA (n=28)**								
W8 DAS28CRP	8/14 (57%)	1/14 (7%)	**17.3 (2.41 to 361.6)**	**0.0029**	**17.3 (2.41 to 361.6)**	**0.0029**	/	/
W8 DAS28ESR	7/16 (44%)	2/12 (17%)	3.9 (0.71 to 30.9)	0.120	2.13 (0.14 to 33.4)	0.589	Age: 1.08 (1.01 to 1.17)	**0.013**
							Disease duration: 0.96 (0.88 to 0.99)	**0.031**
W8 SDAI	9/23 (39%)	0/5 (0%)	N/A (0–/)	0.999	N/A (0–/)	0.999	/	/
W8 CDAI	9/23 (39%)	0/5 (0%)	N/A (0–/)	0.999	N/A (0–/)	0.999	/	/

Bold values denotes stastical significance at the p<0.05 level.
*Number and percentage of anti-UH-RA.305/318/329 antibody positive baseline samples from patients who did (rem) or did not (non-rem) reach remission at week 8 according to different disease activity measures.
†Univariate model for prediction of non-rem using baseline anti-UH-RA.305/318/329 antibody reactivity. A p<0.05 was considered to be statistically significant, and the 95% CI was analysed.
‡Multivariate model for prediction of non-rem using baseline anti-UH-RA.305/318/329 antibody reactivity, corrected for age, gender, RF/ACPA status, disease duration and treatment type.
§Covariate that is found to be significant in predicting non-remission using a certain disease activity measure.
¶ Analysis based on all tested individual baseline RA samples with available clinical data on early remission.
**Analysis based on all tested individual baseline RA samples, which are seronegative for RF and ACPA.
ACPA, anti-citrullinated protein antibodies; BL, baseline; CDAI, Clinical Disease Activity Index; DAS28CRP, Disease Activity Score in 28 joints with C-reactive protein; DAS28ESR, DAS28 with erythrocyte sedimentation rate; HR, high risk; LR, low risk; non-rem, patient not reaching disease remission; OR, Odds Ratio; RA, rheumatoid arthritis; rem, patient reaching disease remission; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; UH, University Hasselt; W, week.

largest improvement in DAS28CRP over the first 16 weeks. Baseline samples were taken before first-line therapy initiation. Detailed explanation of the procedure is described in online supplemental methods. Demographic and clinical parameters' details at baseline and week 16, of the 40 patients used in the SAS screening, are shown in online supplemental table 2.

Identifying antibody reactivity in pooled and individual baseline samples

The output of the SAS was analysed and the enrichment and identity of the selected individual antigens was determined by colony PCR, DNA fingerprinting and sequencing, as explained in online supplemental methods. The fingerprinting analysis identified 41 different enriched patterns, which were encountered more than once and hence were common patterns. Subsequently, antibody reactivity towards 41 identified University Hasselt (UH)-RA antigens (UH-RA.301 until UH-RA.341) was determined in pooled baseline samples by phage ELISA as described previously[12] and in online supplemental methods, in order to select antigens with increased antibody reactivity in DAS28CRP non-rem samples. To this end, 4 serum pools of week 16 DAS28CRP non-rem patients (10 patients/pool; 20 patients from the non-rem SAS pool and 20 additional non-rem patients), and 5 serum pools of week 16 DAS28CRP rem patients (10 patients/pool; 20 patients from rem SAS pool and 30 additional rem patients) were used (figure 1 and demographics in online supplemental table 2).

Based on the differential reactivity in the non-rem pools compared with the rem pools, the 41 anti-UH-RA antigens were ranked. Six UH-RA antigens with the

highest antibody reactivity in >1 non-rem pools and low reactivity in the rem pools were selected. Antibody reactivity against these six antigens was determined using phage ELISA, as previously described[12] (online supplemental methods), in individual baseline samples from 136 new additional CareRA trial participants. These were new patients who were not included in the SAS pool and plasma pools (figure 1, online supplemental table 2). 86 age-matched and sex-matched HC were also included. The distribution of patients who did/did not reach early remission/LDA, or sustained remission/LDA, is shown in online supplemental table 3.

Competition ELISA and epitope mapping

To confirm the phage-displayed peptide as the actual target of the observed antibody reactivity, competition ELISA was performed. Briefly, serum samples were preincubated with increasing concentrations (0–30 µg/mL) of synthetic peptide (>85% purity, GL Biochem, China) (online supplemental table 4) before being used in phage ELISA. Details on both procedures and data analysis are described in online supplemental methods.

Binding of the anti-UH-RA antibodies to synovial tissue and primary fibroblast-like synoviocytes

Anti-UH-RA.305/329 antibodies were purified from plasma samples of RA patients using small-column chromatography (online supplemental methods). In addition, rabbit polyclonal antibodies against UH-RA.329 were obtained (Eurogentec, Belgium). The specificity of purified antibodies against the phage displayed peptide was validated using competition ELISA (online supplemental methods). To identify the tissue expression of the antigenic targets of the anti-UH-RA antibodies, immunofluorescence (IF) was performed on formalin-fixed, paraffin-embedded knee synovial tissue sections from one RA patient using human purified antibodies for UH-RA.305/329. Binding of the identified antibodies to SW982 cells, a fibroblast-like synoviocyte (FLS) cell line, was tested using human purified antibody for UH-RA.305 and human purified antibody or rabbit polyclonal antibody for UH-RA.329. FLS reactivity in the tissue sections and cell line was confirmed by staining for vimentin (Dako, USA) or vimentin and CD90 (Cell Signaling, USA), respectively. Control staining for the antibodies was performed by omitting the primary antibody. The images were collected using Leica DM2000 LED dual-viewing microscope (Leica, Germany) and the results were validated by two pathologists. Detailed methods for the IF procedures are given in online supplemental methods.

Statistical analysis

All samples were tested in duplicate and experiments were performed independently at least twice. To correct for non-specific reactivity, samples were also tested on empty phage without displayed antigen. Samples with an optical density (OD) signal for the empty phage higher than 0.5 were excluded. Results were expressed as the average ratio of antigen-expressing phage OD over empty phage OD. The coefficient of variation for duplicate ODs and for ratios of experimental repeats was lower than 20%. A cut-off for antibody positivity was determined via changepoint analysis in R Studio using the Pruned Exact Linear Time algorithm. This algorithm allows for the division of a series of ascending values into subgroups based on statistical changepoints. The cut-off for antibody positivity was set at five times the SD above the mean ratio (AVG+5 SD) of all non-reactive samples (represented by the lowest subgroup from the changepoint analysis). Data analysis was performed by using JMP, V.17.0.0. Antibody positivity against individual UH-RA antigens was compared between groups by applying Fisher's exact test. To test the probability that anti-UH-RA antibody positivity was greater in the non-rem than in the rem group, the one-tailed test was used. To test the probability that anti-UH-RA antibody positivity was different in RA patients than in HC, the two-tailed test was used. A $p<0.05$ was considered to be statistically significant.

Antibody reactivity against a panel of antigens included antibody positivity for at least one of the antigens included in the panel. Nominal logistic regression was performed to test whether antibody reactivity against the UH-RA.305/318/329 panel could predict non-rem or non-LDA for the DAS28CRP/DAS28ESR/SDAI/CDAI disease activity measures. Tests and CIs on ORs were likelihood ratio (LR)-based. Due to the explorative nature of

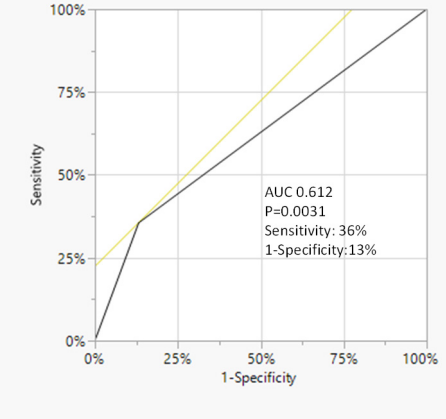

Figure 2 Receiver operating characteristic (ROC) curve for the relationship between failure to achieve remission according to week 8 DAS28CRP in all RA patients and anti-UH-RA.305/318/329 antibody reactivity with age, sex, RF/ACPA seronegativity, treatment type and disease duration as covariates in the multivariate logistic regression. The yellow line in the plot is a straight line at a 45° angle tangent to the ROC curve, and the contact point with the ROC curve shows the optimal cut-off value. A $p<0.05$ was considered to be statistically significant. ACPA, anti-citrullinated protein antibody; AUC, area under the curve; DAS28CRP, Disease Activity Score 28-joint C-reactive protein; RF, rheumatoid factor.

Table 4 Baseline anti-UH-RA.305/318/329 reactivity according to LDA at week 8

Disease activity measure	BL panel reactivity W8 non-LDA*	BL panel reactivity W8 LDA*	Univariate model†		Multivariate model‡		Covariate§	
			OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
All RA (n=135–136)¶								
W8 DAS28CRP	9/29 (31%)	19/107 (18%)	2.08 (0.78 to 5.22)	0.130	1.59 (0.51 to 5.0)	0.424	Treatment type: Tight step up-slim LR: 34.6 (2.91 to 409.8)	**0.005**
							Sex: 3.54 (1.25 to 10.07)	**0.0177**
							Disease duration: 0.97 (0.94 to 0.99)	**0.0105**
							Seronegativity: 4.19 (1.05 to 16.71)	**0.0424**
w8 DAS28ESR	9/33 (27%)	19/102 (19%)	1.64 (0.64 to 4.02)	0.298	1.10 (0.37 to 3.36)	0.854	Age: 1.06 (1.02 to 1.1)	**0.0007**
							Seronegativity: 3.30 (1.27 to 8.65)	**0.015**
W8 SDAI	8/23 (35%)	20/113 (18%)	2.48 (0.9 to 6.56)	0.079	1.77 (0.48 to 6.53)	0.389	Treatment type: Tight step up-slim LR: 60.25 (4.19 to 866)	**0.0026**
							Sex 6.20 (1.78 to 21.6)	**0.0041**
							Disease duration: 0.97 (0.93 to 0.99)	**0.0077**
							Seronegativity: 6.70 (1.48 to 30.4)	**0.0138**
W8 CDAI	9/23 (39%)	19/113 (17%)	**3.18 (1.18 to 8.38)**	**0.023**	2.59 (0.77 to 8.52)	0.118	Treatment type: Tight step up-slim LR: 28.5 (2.5 to 324.6)	**0.0008**
							Sex: 4.04 (1.3 to 13.14)	**0.0136**
							Disease duration: 0.97 (0.93 to 0.99)	**0.0083**
RF/ACPA seronegative RA (n=28)**								
W8 DAS28CRP	6/11 (55%)	3/17 (18%)	**5.6 (1.07 to 35.93)**	**0.041**	3.54 (0.31 to 40.4)	0.308	Treatment type: Tight step up-slim LR: 36.42 (2.59 to 511.4)	**0.001**
							Disease duration 0.97 (0.90 to 0.99)	**0.044**
W8 DAS28ESR	5/12 (42%)	4/16 (25%)	2.1 (0.43 to 11.5)	0.351	1.81 (0.02 to 185.8)	0.801	Treatment type: Tight step up-slim LR: 156 (2.73 to 8930.9)	**0.007**
							Disease duration: 0.85 (0.7 to 0.96)	**0.0005**
							Age: 1.16 (1.04 to 1.39)	**0.0031**
W8 SDAI	6/11 (55%)	3/17 (18%)	**5.6 (1.07 to 35.93)**	**0.041**	3.54 (0.31 to 40.4)	0.308	Treatment type: Tight step up-slim LR: 36.42 (2.59 to 511.4)	**0.001**
							Disease duration: 0.97 (0.90 to 0.99)	**0.0439**

Continued

Table 4 Continued

Disease activity measure	BL panel reactivity W8 non-LDA*	BL panel reactivity W8 LDA*	Univariate model†		Multivariate model‡		Covariates§	
			OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
W8 CDAI	5/10 (50%)	4/18 (22%)	3.5 (0.68 to 20.05)	0.135	2.23 (0.21 to 24.2)	0.51	Treatment type: Tight step up-slim LR: 39.37 (2.71 to 571.3)	**0.0009**
							Disease duration: 0.96 (10.89 to 0.99)	**0.033**

Bold values denotes stastical significance at the p <0.05 level.
*Number and percentage of anti-UH-RA.305/318/329 positive baseline samples from patients who did (LDA) or did not (non-LDA) reach LDA at week 8 according to different disease activity measures.
†Univariate model for prediction of non-LDA using baseline anti-UH-RA.305/318/329 antibody reactivity. A p<0.05 was considered to be statistically significant, and the 95% CI was analysed.
‡Multivariate model for prediction of non-LDA using baseline anti-UH-RA.305/318/329 antibody reactivity, corrected for age, gender, RF/ACPA status, disease duration, and treatment type.
§Covariate that is found to be significant in predicting non-LDA using a certain disease activity measure.
¶Analysis based on all tested individual baseline RA samples with available clinical data on early LDA.
**Analysis based on all tested individual baseline RA samples, which are seronegative for RF and ACPA.
ACPA, anti-citrullinated protein antibodies; BL, baseline; CDAI, Clinical Disease Activity Index; DAS28CRP, Disease Activity Score in 28 joints with C-reactive protein; DAS28ESR, DAS28 with erythrocyte sedimentation rate; LDA, patient reaching low disease activity; non-LDA, patient not reaching low disease activity; OR, Odds Ratio; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; UH, University Hasselt; W, week.

this study, Bonferroni correction was not applied. Hence, a p<0.05 was considered to be statistically significant, and the 95% CI was analysed. In univariate analyses, antibody reactivity against the UH-RA.305/318/329 panel was used as the sole predictor for non-rem or non-LDA. In multivariate analyses, antibody reactivity against the UH-RA.305/318/329 panel, age, sex, RF/ACPA status (as one covariate: considered seronegative if both RF and ACPA antibodies were absent, considered seropositive if one of the antibodies were present), disease duration and treatment type were used as predictors for non-rem or non-LDA. Stepwise-backward selection was applied, and predictors with a p<0.05 were included in the final model. In case of missing follow-up data on the analysed disease activity measure, comparison with baseline antibody reactivity was excluded for that sample. Receiver operating characteristic (ROC) curves were obtained for the significant relationship between failure to achieve remission or LDA according to different disease activity indices and anti-UH-RA.305/318/329 antibody reactivity with age, sex, RF/ACPA seronegativity, treatment type and disease duration as covariates in the multivariate logistic regression.

RESULTS

Identification of novel antibody biomarkers that correlate with failure to achieve early DAS28CRP remission

We previously constructed two cDNA phage display libraries originating from one hip[14] and three knee[15] RA synovia. These libraries have been characterised in previous studies and have been shown to contain cDNA inserts encoding proteins/protein fragments found in RA synovia.[14 15] Additionally, a proportion of the cDNA inserts were characterised by an out-of-frame cDNA translation or the translation of non-coding sequences, representing non-physiological peptides.[15] Combined, these libraries included 1.2×10^7 different antigens, which were screened for antibody reactivity using SAS in baseline sera of RA patients who failed to reach week 16 DAS28CRP remission after first-line combination therapy (figure 1 and online supplemental figure 1). Using this screening, 41 novel UH-RA antigens (UH-RA.301 to UH-RA.341) were identified. Initial antibody reactivity testing against these 41 individual antigens in serum pools of week 16 DAS28CRP non-rem (4 pools) and week 16 DAS28CRP rem (5 pools) patients, showed increased antibody reactivity in >1 non-rem pools and low antibody reactivity in rem pools for 6 antigens (online supplemental figure 2). These antigens, UH-RA.305/308/314/318/329/339, consist of short, non-physiological peptides, between 5 and 37 amino acids in length, which show homology to several human proteins (table 1) expressed in human synovial lymphoid, myeloid or fibroid cells according to the online database from Lewis *et al*,[16] such as superoxide dismutase 2 (SOD2), cardiolipin synthase (CRLS1), interleukin-15 receptor subunit alpha (IL15RA),

Table 5 Baseline anti-UH-RA.305/318/329 reactivity according to sustained disease remission from weeks 8 to 52

Disease activity measure	BL panel reactivity non-sust rem*	BL panel reactivity sust rem*	Univariate model†		Multivariate model‡		Covariate§	
			OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
All RA (n=129–133)¶								
DAS28CRP	21/86 (24%)	6/47 (13%)	2.27 (0.86 to 6.42)	0.10	2.14 (0.77 to 6.65)	0.146	Treatment type: 1. Tight step up-Slim LR: 6.67 (1.31 to 34.0)	**0.016**
							2. Avant garde-classic 3.93 (1.32 to 11.78)	**0.012**
DAS28ESR	21/93 (23%)	5/36 (14%)	1.81 (0.67 to 5.8)	0.256	1.46 (0.47 to 4.51)	0.50	/	/
SDAI	27/114 (24%)	1/19 (5%)	**5.59 (1.07 to 102.8)**	**0.039**	**5.59 (1.07 to 102.8)**	**0.039**	/	/
CDAI	27/114 (24%)	1/19 (5%)	**5.59 (1.07 to 102.8)**	**0.039**	**5.59 (1.07 to 102.8)**	**0.039**	/	/
RF/ACPA seronegative RA (n=26–27)**								
DAS28CRP	8/19 (42%)	1/8 (13%)	5.1 (0.7 to 105.2)	0.115	4.19 (0.51 to 90.5)	0.193	/	/
DAS28ESR	8/20 (40%)	1/6 (17%)	3.33 (0.43 to 70.3)	0.27	1.64 (0.13 to 40.2)	0.704	/	/
SDAI	9/25 (36%)	0/2 (0%)	N/A (0.31– /)	0.192	N/A (0.31– /)	0.192	/	/
CDAI	9/25 (36%)	0/2 (0%)	N/A (0.31– /)	0.192	N/A (0.31– /)	0.192	/	/

Bold values denotes stastical significance at the p<0.05 level.
*Number and percentage of anti-UH-RA.305/318/329 positive baseline samples from patients who did (sust rem) or did not (non-sust rem) reach sustained remission from weeks 8 to 52 according to different disease activity measures.
†Univariate model for prediction of non-sust rem using baseline anti-UH-RA.305/318/329 antibody reactivity. A p<0.05 was considered to be statistically significant, and the 95% CI was analysed.
‡Multivariate model for prediction of non-sust rem using baseline anti-UH-RA.305/318/329 antibody reactivity, corrected for age, gender, RF/ACPA status, disease duration, and treatment type.
§Covariate that is found to be significant in predicting sustained non-remission using a certain disease activity measure.
¶Analysis based on all tested individual baseline RA samples, with available clinical data on sustained remission.
**Analysis based on all tested individual baseline RF/ACPA seronegative RA samples, with available clinical data on sustained remission.
ACPA, anti-citrullinated protein antibodies; BL, baseline; CDAI, Clinical Disease Activity Index; DAS28CRP, Disease Activity Score in 28 joints with C-reactive protein; DAS28ESR, DAS28 with erythrocyte sedimentation rate; N/A, not available; non-sust rem, patient not reaching sustained disease remission; OD, Odds Ratio; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; sust rem, patient reaching sustained disease remission; UH, University Hasselt; W, week.

interleukin-27 receptor subunit alpha (IL27RA) and low-density lipoprotein receptor-related protein 4 (LRP4).

Baseline antibody reactivity against UH-RA antigens and early remission

We examined whether baseline antibody reactivity towards the 6 UH-RA antigens was associated with failure to reach week 8 DAS28CRP remission in 136 new individual samples, which were not included in the SAS and plasma pools (table 2). Baseline anti-UH-RA.305 antibody reactivity was significantly associated with week 8 DAS28CRP remission (LR+3.52, p=0.033). Moreover, antibody reactivity against a final panel of three UH-RA antigens, namely UH-RA.305/318/329, identified 36% of RA patients who failed to reach week 8 DAS28CRP remission, compared with 13% of RA patients who did reach week 8 DAS28CRP remission and thus showed the highest LR+ for not reaching DAS28CRP remission at week 8 (table 2, LR+2.7, p=0.003).

Although this study was not aimed at identifying antibody biomarkers discriminating diseased and control subjects, antibody reactivity against each of the 6 UH-RA antigens was determined in individual baseline samples of the 136 RA patients and 86 HC (online supplemental table 5). Baseline anti-UH-RA.305 antibody reactivity was similar in RA patients and HC, but antibody reactivity against UH-RA.329 or UH-RA.318 was 2.6–2.8 fold higher in RA patients than in HC. For the panel of UH-RA.305/318/329, antibody reactivity was 1.4-fold higher in RA patients than in HC, although not significantly.

Baseline antibody reactivity against UH-RA antigens was associated with early non-remission in total RA and seronegative patients

Next, we studied if there is an association between baseline anti-UH-RA.305/318/329 antibody reactivity and early remission as defined by multiple disease activity measures (table 3). Antibody reactivity against the panel of UH-RA.305/318/329 antigens was defined by combined antibody reactivity against at least one of these antigens. In a univariate model, failure to achieve remission at week 8 according to the DAS28CRP (OR (95% CI) 3.63 (1.54 to 8.76), p=0.0031) or the DAS28ESR (OR (95% CI) 3.13 (1.34 to 7.71), p=0.0082) disease activity measures was associated with baseline anti-UH-RA.305/318/329 antibody reactivity as the sole predictor (table 3). Similarly, in a multivariate model, corrected for age, gender, RF/ACPA status, disease duration and treatment type, anti-UH-RA.305/318/329 antibody reactivity was associated

Table 6 Baseline anti-UH-RA.305/318/329 reactivity according to sustained LDA from weeks 8 to 52

Disease activity measure	BL panel reactivity non-sust LDA*	BL panel reactivity sust LDA*	Univariate model†		Multivariate model‡		Covariate§	
			OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
All RA (n=122–127)¶								
DAS28CRP	18/59 (31%)	8/68 (12%)	**3.29 (1.35 – 8.69)**	**0.009**	**3.70 (1.37 to 10.89)**	**0.009**	Treatment type: 1. Avant garde-classic: 5.57 (1.62 to 19.2)	**0.004**
							2. Slim HR-classic : 3.25 (1 to 10.89)	**0.048**
							Sex: 3.17 (1.34 to 7.99)	**0.008**
DAS28ESR	19/68 (28%)	6/57 (11%)	**3.29 (1.28 to 9.68)**	**0.013**	**2.76 (1.04 to 8.27)**	**0.042**	Age: 1.04 (1.01 to 1.08)	**0.004**
SDAI	14/51 (27%)	11/71 (15%)	2.06 (0.85 to 5.12)	0.109	1.81 (0.67 to 4.97)	0.253	Age: 1.04 (1.01 to 1.07)	**0.015**
CDAI	15/53 (28%)	10/69 (14%)	2.33 (0.96 to 5.87)	0.062	2.08 (0.84 to 5.32)	0.114	Age: 1.03 (1.0 to 1.06)	**0.035**
RF/ACPA seronegative RA (n=24–25)**								
DAS28CRP	7/13 (54%)	1/12 (8%)	**12.83 (1.71 to 271.5)**	**0.011**	4.49 (0.33 to 148.7)	0.27	Treatment type: Tight step up-Slim LR: 97.4 (13.54 to /)	**0.0008**
							Disease duration: 0.97 (0.84 to 0.99)	**0.015**
							Sex: 28.5 (1.14 to /)	**0.42**
DAS28ESR	7/16 (44%)	1/8 (13%)	5.44 (0.72 to 114.5)	0.107	1.16(0.04 to 22.3)	0.92	/	/
SDAI	7/13 (54%)	1/12 (8%)	**12.83 (1.71 to 271.5)**	**0.011**	4.49 (0.33 to 148.7)	0.27	Treatment type: Tight step up-slim LR: 97.4 (13.54 to /)	**0.0008**
							Disease duration: 0.97 (0.84 to 0.99)	**0.015**
							Sex: 28.5 (1.14 to /)	**0.42**
CDAI	6/12 (50%)	2/13 (15%)	5.5 (0.94 to 46.5)	0.0597	4.91 (0.33 to 214.3)	0.26	Treatment type: Tight step up-slim LR: 167 (4.29 to /)	**0.0004**
							Disease duration: 0.92 (0.82 to 0.99)	**0.0074**
							Sex: 47 (1.33 to /)	**0.03**

Bold values denotes stastical significance at the p<0.05 level.
*Number and percentage of anti-UH-RA.305/318/329 positive baseline samples from patients who did (sust LDA) or did not (non-sust LDA) reach sustained LDA from weeks 8 to 52 according to different disease activity measures.
†Univariate model for prediction of non-sust LDA using baseline anti-UH-RA.305/318/329 antibody reactivity. A p<0.05 was considered to be statistically significant, and the 95% CI was analysed.
‡Multivariate model for prediction of non-sust LDA using baseline anti-UH-RA.305/318/329 antibody reactivity, corrected for age, gender, RF/ACPA status, disease duration, and treatment type.
§Covariate that is found to be significant in predicting non-sust LDA using a certain disease activity measure.
¶Analysis based on all tested individual baseline RA samples, with available clinical data on sustained LDA.
**Analysis based on all tested individual baseline RF/ACPA seronegative RA samples, with available clinical data on sustained LDA.
ACPA, anti-citrullinated protein antibodies; BL, baseline; CDAI, Clinical Disease Activity Index; DAS28CRP, Disease Activity Score in 28 joints with C-reactive protein; DAS28ESR, DAS28 with erythrocyte sedimentation rate; LDA, low disease activity; non-sust LDA, patient not reaching sustained LDA; OD, Odds Ratio; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; sust LDA, patient reaching sustained LDA; UH, University Hasselt; W, week.

with failure to reach DAS28CRP (OR (95% CI) 3.63 (1.54 to 8.76), p=0.0031) or DAS28ESR (OR (95% CI) 2.92 (1.22 to 7.31), p=0.016) remission in all RA patients. Old age was a significant covariate that was associated with this week 8 DAS28ESR non-rem. Furthermore, the sensitivity and specificity of this panel for predicting non-remission after first-line therapy according to the week 8 DAS28CRP were 36% and 87%, respectively, as shown in the corresponding ROC curve (figure 2).

Although a similar trend was observed for remission according to the more stringent SDAI and CDAI disease activity measures, baseline anti-UH-RA.305/318/329 antibody reactivity was not significantly associated with early SDAI or CDAI remission. Treatment type was a covariate that was significantly associated with week 8 SDAI or CDAI remission (details about the specific treatment type that was significant for each disease activity index are shown in table 3). Additionally, seronegativity was also a significant covariate for week 8 CDAI remission.

In RF/ACPA-seronegative patients, baseline anti-UH-RA.305/318/329 antibody reactivity identified 57% of these patients who failed to reach week 8 DAS28CRP rem, compared with 7% in patients who did, and was associated with this disease activity measure in both the univariate and multivariate models (OR (95% CI) 17.3 (2.41 to 361.6), p=0.0029) (table 3).

Baseline antibody reactivity against UH-RA antigens was associated with early non-LDA in seronegative patients

Baseline antibody reactivity against the UH-RA.305/318/329 antigens was present in 39% of all RA patients and 55% of seronegative RA patients who failed to achieve week 8 LDA in comparison to 17% of all RA patients and 18% of seronegative patients who did according to CDAI and DAS28CRP/SDAI, respectively, and it was also associated with the LDA states in the univariate analysis (week 8 CDAI LDA (OR(95% CI) 3.18 (1.18 to 8.38),p=0.023 in all RA patients) and week 8 DAS28CRP and week 8 SDAI LDA (OR(95% CI) 5.6 (1.07 to 35.93), p=0.041, same OR for both in seronegative patients), table 4). However, this could not be confirmed in the multivariate model. Hereby, treatment type and disease duration were significant covariates for all week 8 LDA disease activity indices in all RA patients (except for week 8 DAS28ESR LDA) and RF/ACPA seronegative patients (details about the specific treatment type that was significant for each disease activity index is shown in table 4). Old age was a significant covariate associated with week 8 DAS28ESR LDA in all RA and RF/ACPA seronegative patients. Sex, particularly being a male, was a significant covariate for week 8 DAS28CRP LDA, week 8 SDAI LDA and week 8 CDAI LDA in all RA patients. For week 8 DAS28CRP/DAS28ESR and SDAI LDA, RF/ACPA seronegativity was a significant covariate in all RA patients (specific p values and 95% CI for these covariate measures are shown in table 4).

Baseline antibodies against UH-RA antigens were associated with sustained non-remission and non-LDA

Next, the predictive potential of baseline anti-UH-RA.305/318/329 antibody reactivity for sustained remission and LDA was identified in 24% of RA patients who failed to achieve sustained remission in comparison to 5% of RA patients who did according to SDAI and CDAI, and was associated with failure to achieve sustained remission (SDAI & CDAI non-sust rem OR(95% CI) 5.59 (1.07 to 102.8), p=0.039 for both univariate and multivariate model, table 5). Treatment type was a significant covariate for DAS28CRP non-sust rem. In RF/ACPA-seronegative patients, a similar trend could be observed, although not statistically significant. Finally, anti-UH-RA.305/318/329 baseline antibody reactivity was able to identify 28%–31% of all RA patients who failed to reach sustained LDA in comparison to 11%–12% of all RA patients who did according to DAS28ESR and DAS28CRP, respectively (table 6). This antibody reactivity was also associated with failure to reach sustained DAS28CRP and DAS28ESR LDA in both the univariate (non-sust DAS28CRP LDA OR(95% CI) 3.29 (1.35 to 8.69), p=0.009 and non-sust DAS28ESR LDA OR (95% CI) 3.29 (1.28 to 9.68), p=0.013) and multivariate analysis (non-sust DAS28CRP LDA OR(95% CI) 3.7 (1.37 to 10.89), p=0.009 and non-sust DAS28ESR LDA OR (95% CI) 2.76 (1.04 to 8.27), p=0.042) in all RA patients (table 6). Treatment type and sex (male) were significant covariates for the non-sust DAS28CRP LDA while old age was a significant covariate for non-sust DAS28ESR, SDAI, CDAI LDA (table 6). In RF/ACPA-seronegative patients, baseline anti-UH-RA.305/318/329 antibody reactivity was present in 54% of patients failing to reach DAS28CRP and SDAI sustained LDA in comparison to 8% of patients reaching DAS28CRP and SDAI sustained LDA and was associated with these states in the univariate model (OR(95% CI) 12.83 (1.71 to 271.5),p=0.011). Disease duration, sex and treatment type were significant covariates that was associated with sustained DAS28CRP, SDAI and CDAI non-LDA in seronegative patients in the multivariate analysis.

Graphical representation of the ROC curves of the significant results of the multivariate analysis is shown in online supplemental figure 3.

Anti-UH-RA antigens are mimotopes identified through epitope mapping

The original epitope that elicited the antibody response in the RA patients who tested positive for the anti-UH-RA antigens is still unknown. To that end, epitope mapping using competition ELISA with synthetic peptides (online supplemental table 4) was performed to determine the exact antigen sequence anti-UH-RA antibodies bind to. Epitope mapping using competition ELISA with synthetic peptides could define the epitope sequences recognised by anti-UH-RA.305, and anti-UH-RA.329 antibodies, to the antigen sequences indicated in online supplemental table 4 (online supplemental figue 4A–C). For the longer UH-RA.318 antigen, competition ELISA could show that the epitope recognised by anti-UH-RA.318 antibodies is part of the UH-RA antigen sequence indicated in online supplemental table 4 (online supplemental figure 4B).

FLS is the target cell type for anti-UH-RA antibodies

We succeeded in isolating two of the anti-UH-RA antibodies, anti-UH-RA.305/329 antibody, from human serum and we obtained rabbit polyclonal antibodies for anti-UH-RA.329 antibody. The specificity of the purified antibodies for the corresponding synthetic peptides was determined using competition ELISA. For each synthetic peptide, competition with the specific phage (UH-RA.305 or UH-RA.329) phage could be demonstrated, whereas preincubation with the control peptide did not affect antibody reactivity (online supplemental figure 5). Thus, these results confirm that these peptides contain the epitopes recognised by the purified antibodies. Additionally, the expression of the anti-UH-RA.305/329 antibody targets in RA synovial tissue was studied using IF. The human anti-UH-RA.305/329 antibodies were able to target cells in the RA synovial tissue, particularly FLS as shown by costaining with vimentin, which is a marker for FLS (figure 3A,B). Validation of the FLS reactivity of anti-UH-RA.305/329 antibodies was done by staining SW982 cells, which is an FLS cell line as demonstrated by its staining for vimentin and CD90 (figure 4A–D, negative controls are shown in online supplemental figure

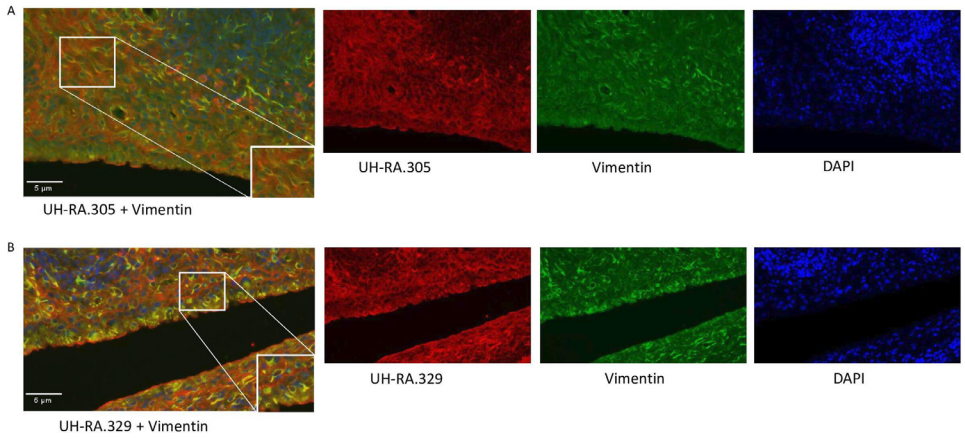

Figure 3 Representative staining of anti-UH-RA.305 and anti-UH-RA.329 antibodies in synovial RA tissue. IF showing colocalization of vimentin (green, FLS marker) and UH-RA.305 (A) or UH-RA.329 (B) (red, using human purified Ab). Magnification ×20, scale bar represents 5 µm. FLS, fibroblast-like synoviocyte; IF, immunofluorescence; RA, rheumatoid arthritis; UH, University Hasselt.

6). Indeed, human anti-UH-RA.305 antibody, human anti-UH-RA.329 antibody and rabbit polyclonal anti-UH-RA.329 antibody showed staining of SW982 cells, confirming FLS as their cellular target. Antigen specificity of the rabbit anti-UH-RA.329 antibody signal was validated by peptide block using UH-RA.329 peptide (online supplemental figure 7). Hence, anti-UH-RA.305 and anti-UH-RA.329 antibodies target FLS in synovial tissue, which indicates the possible biological relevance of these autoantibodies next to their biomarker potential.

DISCUSSION

We identified three novel antibody biomarkers that are associated with failure to achieve remission or LDA after first-line RA therapy. The presence of antibody reactivity against the UH-RA.305, UH-RA.318 or UH-RA.329 antigens before therapy initiation was associated with failure to reach early or sustained disease remission or LDA after the initiation of csDMARD combination therapy. FLSs were shown to be the cellular target of two of these antibodies, namely anti-UH-RA.305 and anti-UH-RA.329 antibodies.

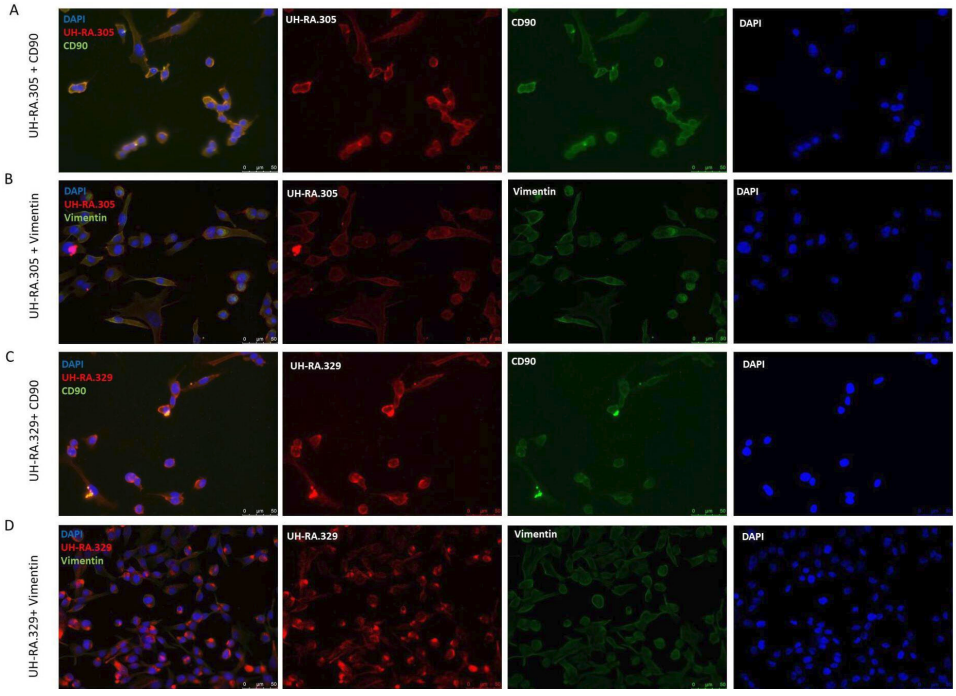

Figure 4 Representative staining of anti-UH-RA.305 and anti-UH-RA.329 antibodies in SW982 cells. IF showing costaining of (A) UH-RA.305 (red, using human purified Ab) and CD90 (green, FLS marker), (B) UH-RA.305 (red, using human purified Ab) and vimentin (green), (C) UH-RA.329 (red, using human purified Ab) and CD90 (green) and (D) UH-RA.329 (red, using rabbit polyclonal Ab) and vimentin (green) in SW982 cells. Magnification ×40, scale bar represents 50 µm. Ab, antibody; FLS, fibroblast-like synoviocyte; IF, immunofluorescence; RA, rheumatoid arthritis; UH, University Hasselt.

A clear improvement of disease activity in the first 3 months after treatment initiation has been shown to be a strong indicator of subsequent treatment success as patients who improve to remission or LDA after 3 months are much more likely to maintain this disease state, than patients who do not.[17] Testing for baseline antibody reactivity against the UH-RA.305/318/329 antigens identified about 35% of RA patients who will not reach DAS28 LDA or remission already at early time points such as week 8, making them a possible valuable baseline predictor of early treatment failure. In ACPA-seronegative RA, it is especially important to contain the disease in an early phase, as it can be strongly associated with long-term sustained DMARD-free remission.[18] As antibody reactivity against the UH-RA antigens could identify an even larger proportion of RF/ACPA-seronegative patients failing to reach early remission or LDA, this could be an important tool to identify seronegative patients who are less likely to respond to first-line therapy.

This study tested whether antibody reactivity against UH-RA.305/318/329 is associated with failure to reach remission/LDA according to various disease activity measures using a multivariate model including age, sex, RF/ACPA status, treatment types, and disease duration as covariates. These covariates are commonly included in prediction models of therapy response. Age and RF/ACPA status were significant covariates associated with failure to reach several early and sustained disease activity indices. Age is usually associated with comorbidities and higher risks of infection; therefore, it is expected that older people would be less likely to be good responders to MTX. However, some studies reported no effect of age on MTX therapy response[19 20] while other studies showed that young age was associated with lack of MTX response.[21 22] Similarly, our results show that old age was associated with failure to reach early and sustained remission or LDA. Besides the diagnostic characteristics of RF and ACPA, these autoantibodies have also been investigated in predicting therapy response. Numerous studies have shown that the presence of RF or ACPA antibodies does not predict MTX response.[21 23–26] On the other hand, several studies reported conflicting roles of these antibodies in predicting therapy response to MTX.[27–30] Few studies have investigated the effect of combined positivity of these autoantibodies on therapy response. One such study showed that RF/ACPA double-positivity was associated with improved CDAI and sustained remission.[31] Here, we showed that RF/ACPA-negativity was associated with failure to reach early LDA. Furthermore, female sex has been associated with MTX failure in a number of studies.[32–34] However, a retrospective study showed that sex was not significantly associated with inadequate response to MTX for early RA.[35] In contrast, in our study, being a male was associated with failure to reach early or sustained LDA.

The current recommendation for RA treatment is to initiate therapy as soon as possible, adopting intensive treatment strategies. MTX is considered to be the anchor drug for RA treatment, however, whether other DMARDs should be added to the treatment strategy is still under debate. Additionally, the optimal doses and the safety of such treatment strategies need to be further investigated. In the CareRA trial, different treatment regimens, based on the COBRA strategy were evaluated. Based on both a 2-year and 5-year evaluation, all regimens combining DMARDs with GCs were equally effective.[4 7] In our study, the treatment type was a predictor for failure to reach early (week 8) and sustained (weeks 8–52) remission/LDA based on various disease activity indices. Treatment type was a significant covariate for lack of week 8 SDAI and CDAI remission in all RA patients. This covariate was also a significant factor associated with week 8 LDA according to the various disease activity indices. Furthermore, treatment type was associated with sustained non-remission as well as sustained non-LDA according to the DAS28CRP in all RA patient and sustained non-LDA according to the DAS28CRP, SDAI and CDAI indices in seronegative patients.

Short disease duration has been associated with remission and LDA. Studies have shown that short disease duration is associated with achievement of remission, including sustained remission.[33 36 37] Earlier identification of RA leads to earlier therapeutic intervention, resulting in rapid control of disease progression. Our results show that the chance of not reaching remission/LDA decreases with disease duration. This can be due to the increased possibility of reaching remission/LDA as time progresses. These results combined highlight the importance of including covariates that can affect therapy response in RA as this reduces bias in the prediction model. Additionally, identifying several covariates such as sex, age, RF/ACPA status, treatment type, and disease duration in therapy response in RA emphasises the multifactorial nature of the prediction model.

Characterisation of the anti-UH-RA antibodies in terms of identity and tissue localisation can contribute to the understanding of the role of these antibodies in therapy response and can identify targets for precision medicine. The anti-UH-RA.305/318/329 responses identified in this study were directed against non-physiological peptide antigens. These sequences probably form mimotopes, or sequences that mimic the *in vivo* antigen these antibodies were originally formed against. For the UH-RA antigens, these are currently unknown, however, they show partial amino acid homology with several human synovial proteins (table 1 and Lewis *et al*[16]), some of which are potentially relevant targets in RA. These include the cytokine receptor subunits IL15RA[38] and IL27RA,[39] LRP4, which are involved in bone metabolism,[40 41] SOD2, a regulator of oxidative stress, which can be linked to RA pathology and therapy response,[42] and CRLS1, which is involved in inflammation.[43] On the other hand, immunofluorescent analysis of the tissue expression of the UH-RA.305/329 antigens revealed that they target FLS cell line and FLS in the synovial tissue of RA patients. In RA, FLSs and macrophages are the dominant cell types in the synovium and are key players in the destructive process of the disease.[44] These cells produce proinflammatory cytokines, attach to and invade articular cartilage, stimulate angiogenesis and contribute to bone erosion.[45]

The results of this study should be interpreted while considering some limitations. With regard to the use of the different indices for disease activity, we have mainly focused on the achievement of remission or LDA compared with the cut-offs for these outcomes, not considering the level of improvement of disease activity. Still, achieving a state of remission or LDA in an early phase of therapy has been shown to be a more valuable indicator than the response compared with baseline.[17] Future research should be carried out in order to determine whether the anti-UH-RA antibodies are merely associated with a lack of therapy response or also play an active part in this response or disease pathology. Another limitation is the low number of antibody positive patients in some groups, however, further validation of the presence of these antibodies in a larger number of patients from different RA study populations will be conducted. Furthermore, the observed antibody reactivity is measured against mimotope antigens. As the *in vivo* antigens are still unknown, this limits the interpretation of the biological processes that might underlie the reduced likelihood for therapy response in anti-UH-RA.305/318/329 positive patients. Finally, anti-UH-RA.318 could not be purified from human plasma using small chromatography, thus, we could not determine the cellular target of this antibody. In conclusion, baseline antibody reactivity against the panel of UH-RA.305/318/329 antigens is associated with patients who are much less likely to respond to csDMARDs as a first-line treatment. Furthermore, these antibodies would be most effective when included in a panel of multiple biomarkers, instead of functioning as standalone biomarkers. Such a panel, combining various biomarkers, would deliver a more detailed and accurate evaluation of therapy response in RA which could significantly enhance precision medicine and guide more personalised treatment strategies.

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Competing interests PVandormael, VS and PVerschueren have a patent application filed on the biomarkers described in this report.

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