Supplementary Methods

Western blot experiments

Citrullination of human recombinant vimentin was performed as previously described. [7] Carbamylation of human recombinant vimentin was achieved by incubation at 37˚C with 100 mM potassium cyanate for 2 hours, followed by dialysis against phosphate-buffered saline at pH 7.4 at 4˚C to remove excess cyanate. Conjugation of the synthesized acetylated vimentin peptide to carrier albumin was achieved via an N-terminal cysteine or C-terminal cysteine amide residue added to the peptide's sequence. Crosslinking between the peptide and the carrier was performed by an MBS-mediated (Maleimidobenzoyl-N-hydroxysuccinimide ester) optimized coupling procedure according to the JPT Peptide Technologies (Berlin, Germany) protocol. Mass spectrometry of the purified sample confirmed the most abundant protein product corresponded to a 4:1 covalent complex of albumin with the acetylated vimentin peptide, and reverse phase HPLC analysis of purified sample confirmed the removal of essentially all unbound (free) maleimido derivative.

Samples of each modified vimentin protein containing 5 µg of protein were mixed with an equal volume of 2× laemmli sample buffer, boiled and separated by SDS-PAGE (4-12% NuPAGE, Invitrogen). Proteins were transferred to nitrocellulose membranes. Blots were blocked in blocking buffer (PBS containing 5% non-fat dried milk and 0.1% NP-40) for one hour at room temperature and incubated overnight at 4°C with the antibody of interest diluted in blocking buffer. Sera from RA patients (diluted 1:1000) were left untreated or preincubated with 10 µg/ml of the indicated peptide for 2 hours at room temperature, followed by incubation for at least 8 hours at 4°C with nitrocellulose membrane. After washing with blocking buffer, bound antibodies were detected by incubation with Horseradish-peroxidase conjugated secondary anti-human IgG antibody (Dianova, Germany) and the blots were developed using the ECL system (GE Healthcare, Germany).

Vimentin-peptide ELISA

The 12mer peptides were synthesized, in accordance with Fmoc-chemistry at the Peptide Specialty of JPT PeptideTechnologies (Berlin, Germany). An N-terminal extension of the peptides (Biotin-SGSG) was designed for a defined flexibility and to incorporate an affinity tag. Crude fractions after peptide synthesis were purified using high-performance liquid chromatography. Quality and purity of the peptide was assessed by mass spectrometry and analytical high-performance liquid chromatography in according to the manufacture procedure (JPT Peptide Technology; Berlin Germany).

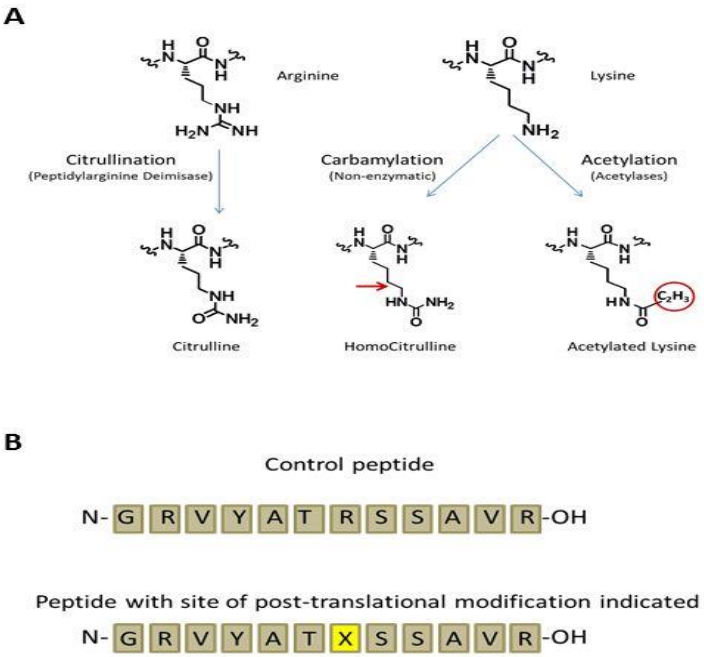
Microtiter plates (Maxisorb, Nunc, Denmark) were precoated with 2 µg/ml streptavidin (Perbio Science Deutschland GmbH, Bonn, Germany) in phosphate-buffered saline (pH7.6). After a 2 hour incubation at 25°C, the plates were blocked with 1% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature. Theses plates were used for binding of the biotinylated peptides/antigens. The biotinylated 12-mer vimentin peptides at a concentration of 0.5 µg/ml were diluted in phosphate-buffered saline(pH7.6) and incubated overnight at 4°C (100 µl/ well). Possible overpluses of peptides/antigens were eliminated by washing with 200µl/well of 0.1% Tween 20 in phosphate-buffered saline. Finally, flicking and slapping removed any residual solution.

The assay was performed in accordance with the general protocol for the Orgentec ELISA system.
In brief, serum samples were diluted 1:101 in sample buffer (phosphate-buffered saline, containing bovine serum albumin and Tween), added to the wells and then incubated for 30 minutes (100 µl/ well). After three washing steps (200 µl/well), horseradish peroxidase conjugated anti-human IgG or anti-human IgG as indicated (Dianova, Hamburg, Germany) was added and incubated for 30 minutes (100 µl/well). Visualization was done by incubation with 3,3',5,5'-tetra-methyl benzidine substrate (TMB) for 15 minutes (100 µl/well), and the reaction was terminated by adding 100 µl stop solution (0.5 mol/l H_2SO_4) to each well. Finally, the absorbance was determined using an ELISA reader

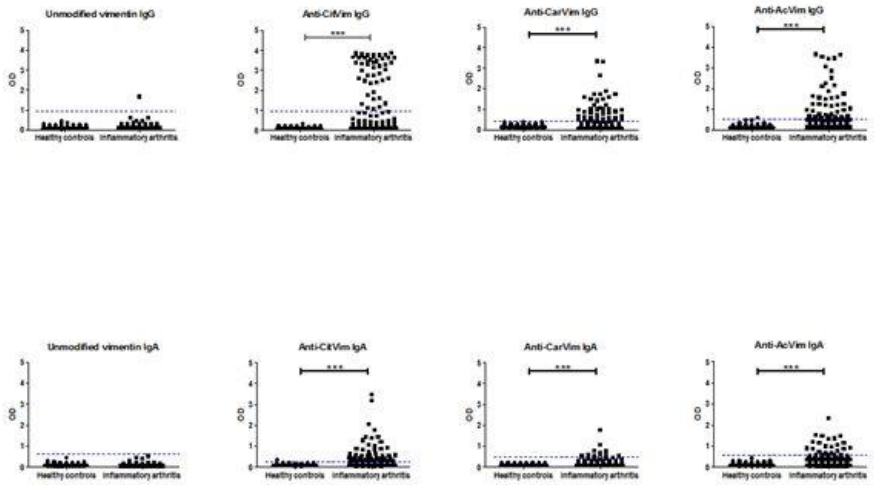
(Rainbow Reader, Tecan). All steps were carried out at room temperature. Background OD was obtained by adding serum to a well without biotinylated peptide.

Supplementary figure legends

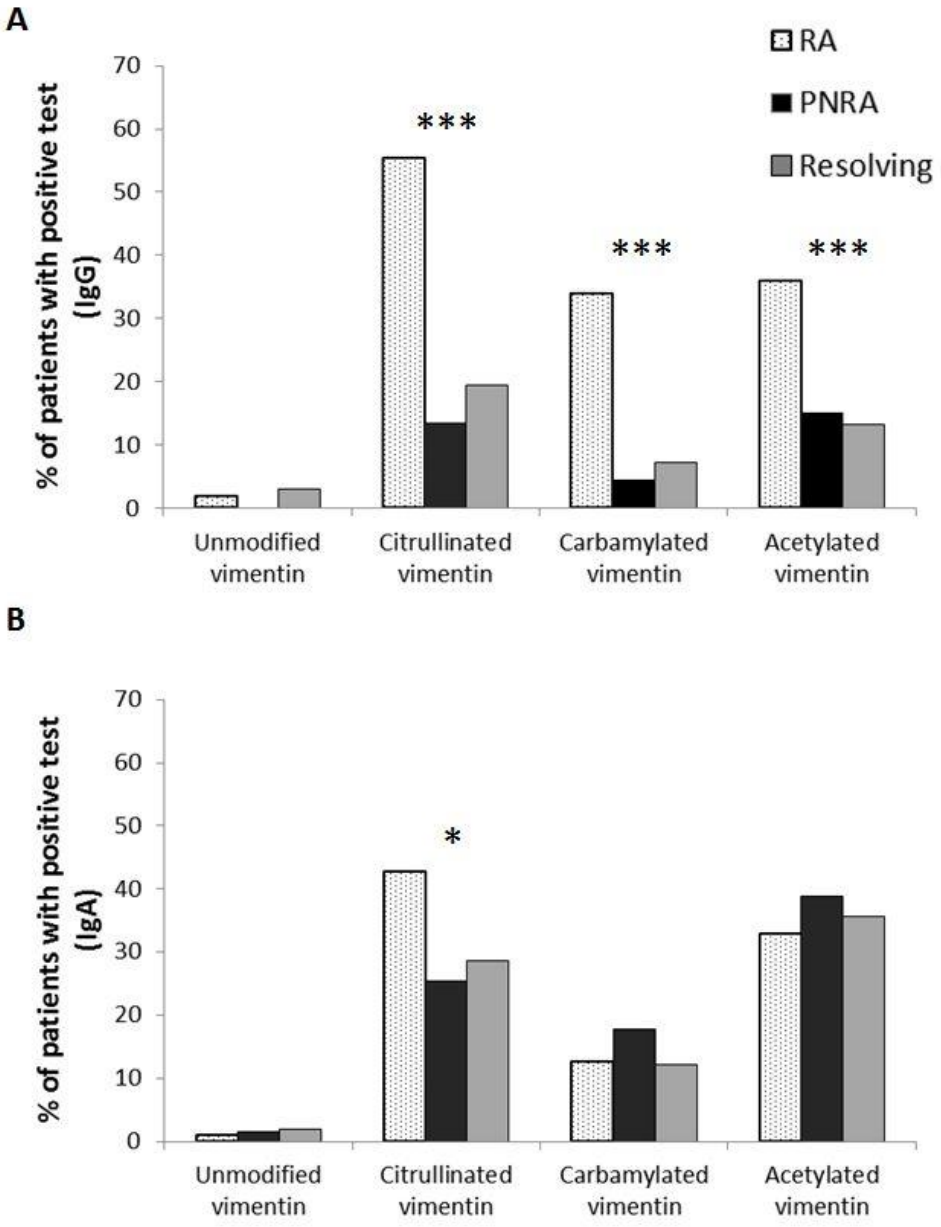
Supplementary Figure 1. A: Illustration of arginine citrullination and lysine carbamylation and acetylation. These post-translationally modified peptides are very similar, with only a few differences between them that are indicated in red. Red arrow indicating how citrulline and homocitrulline differ by an extra carbon atom that makes the homocitrulline side-chain longer. Red circle indicating addition of an acetyl group to lysine instead of an amino group. Acetylated lysine differs from homocitrulline only by one hydrogen and one nitrogen that is exchanged by carbon. B: Schematic representation of the peptide sequence used in the immunometric enzyme immunoassay. The control peptide sequence, derived from native unmodified vimentin is shown on top. Underneath the same sequence, except at one amino acid at position 7 (denoted by X) where post-translational modifications were introduced.



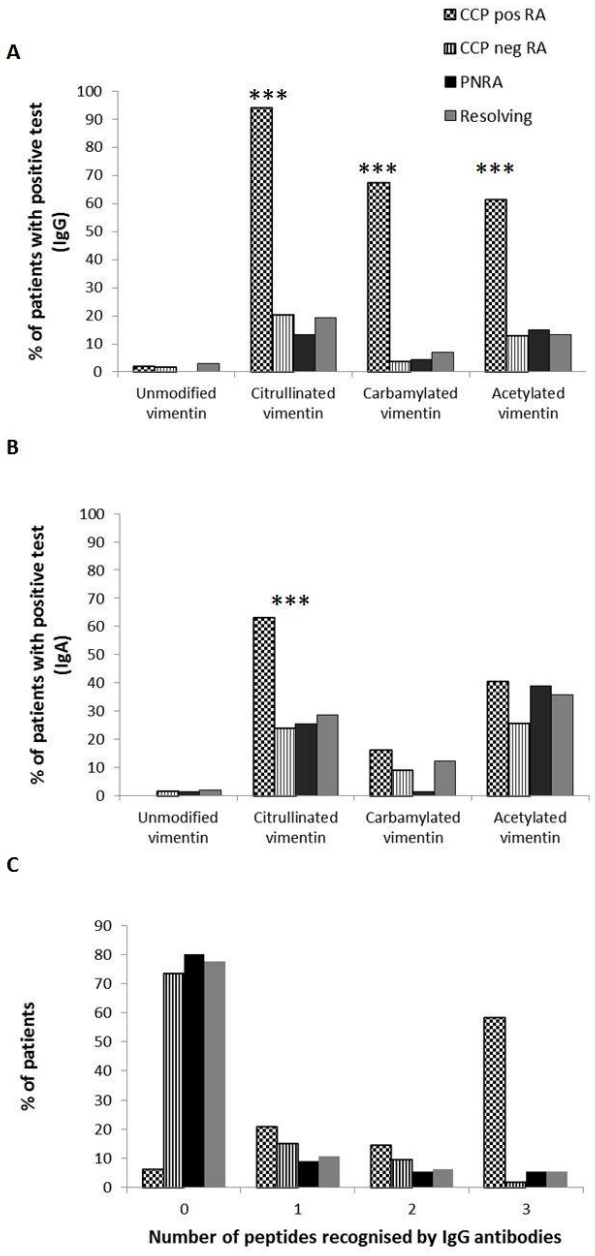
Supplementary Figure 2. Distribution of antibody levels in healthy controls (n=160) and early inflammatory arthritis patients (n=268). Antibody levels expressed as optical density (OD). OD values > 98th percentile of the healthy controls (represented by dotted horizontal line) were considered positive for all antibodies. **p<0.01, ***p≤0.001.



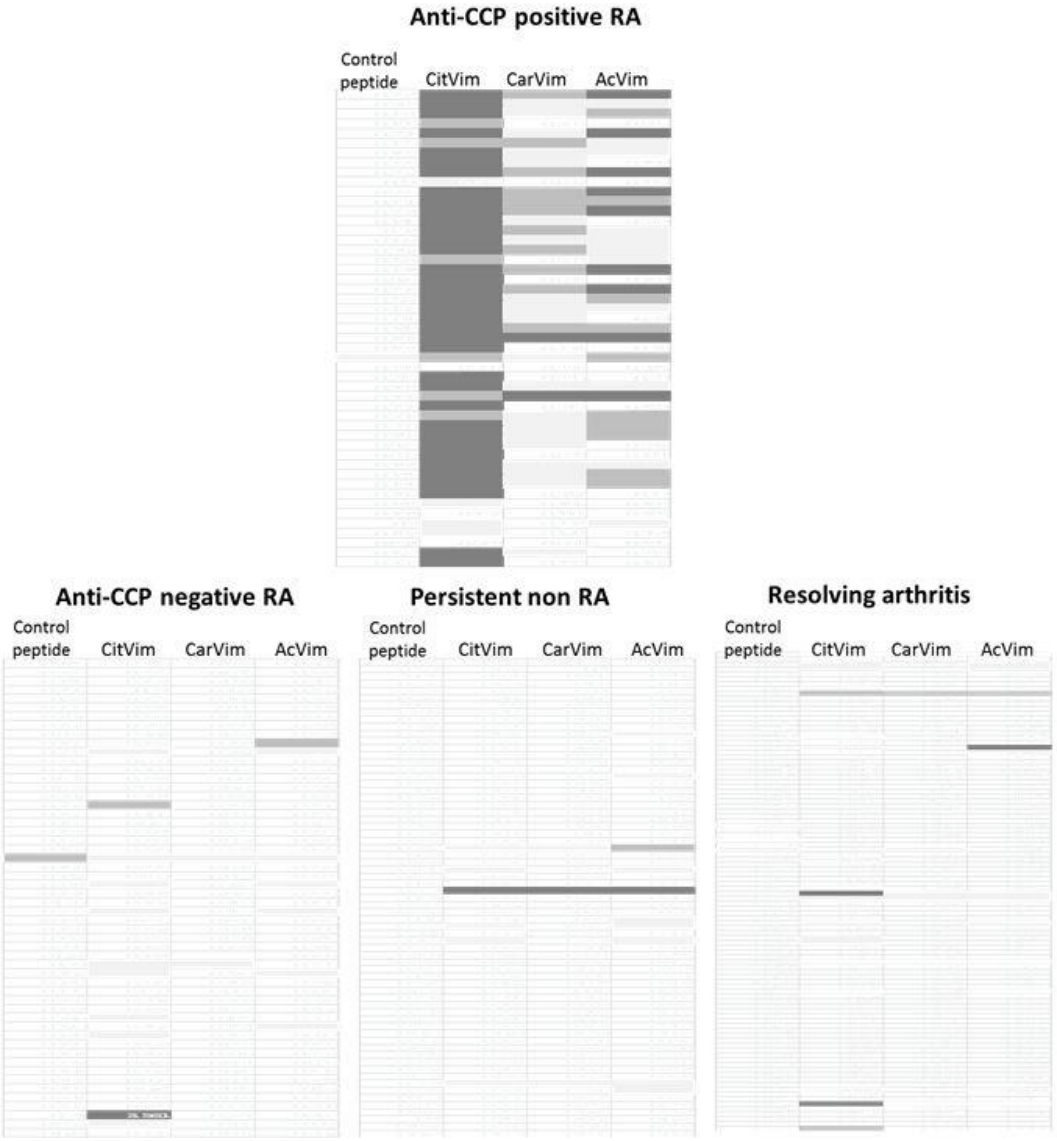
Supplementary Figure 3 Frequency of seropositivity for IgG and IgA antibodies against different post-translationally modified peptides in RA (dotted bars), persistent non RA (black bars) and resolving (grey bars) groups. A: IgG antibody panel, B: IgA antibody panel. *p<0.05, ***p≤0.001. Patients classified according to 2010 ACR EULAR classification criteria for RA.



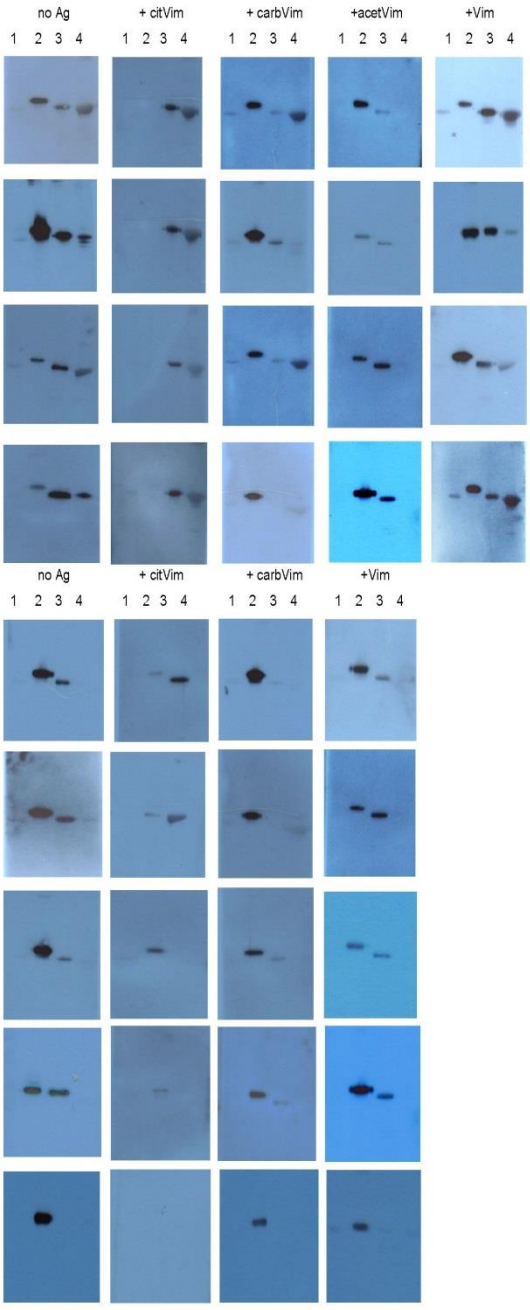
Supplementary Figure 4. Frequency of antibody positivity to different post-translationally modified peptides in each outcome group. Anti-CCP positive RA (checkered bar), anti-CCP negative RA (vertical lines bar), persistent non-RA arthritis (black bar) and resolving arthritis (grey bar). A: IgG reactivities: statistically significant differences in the percentages of patients with positive antibodies were observed between anti-CCP positive RA patients and the remaining groups for all peptides. B: IgA reactivities: statistically significant differences in the frequency of anti-CitVimentin IgA antibody reactivities seen between anti-CCP positive RA patients and the remaining groups but not for the remaining post-translationally modified peptides. ***p≤0.001. C: Distribution of the number of peptides recognised according to outcome group. The highest proportion of anti-CCP positive RA patients recognised 3 peptides whilst the highest proportion of patients in the remaining outcome groups did not recognise any peptides. Patients classified according to 2010 ACR EULAR classification criteria for RA.



Supplementary Figure 5. Graphical representation of IgG antibody reactivity results. Anti-CCP positive RA patients show a distinct antibody profile that differs from that of the remaining outcome groups. This patient group has reactivities against a higher number of peptides and the antibody levels against those peptides are also higher. Shaded squares represent positive tests: light grey: low level positivity (OD: >98th percentile to 1.0), dark grey: medium positivity (OD: >1.0 to 1.9), black: high positivity (OD: >1.9). Each row represents an individual patient, each column represents a peptide. Control: native unmodified vimentin peptide; CitVim: citrullinated vimentin peptide; CarVim: carbamylated vimentin peptide; AcVim: acetylated vimentin peptide. Patients classified according to 2010 ACR EULAR classification criteria for RA.



Supplementary Figure 6. Western blots where unmodified and modified full length human recombinant vimentin proteins have been separated by SDS-PAGE and probed with triple reactive (n=4) and double reactive (n=5) patient sera. Each row of western blots represents a patient. No Ag: blot probed with patient sera not preincubated with any peptides; +citVim: blot probed with patient sera preincubated with citrullinated vimentin peptide; +carbVim: blot probed with patient sera preincubated with carbamylated vimentin peptide; +acetVim: : blot probed with patient sera preincubated with acetylated vimentin peptide; +Vim: blot probed with patient sera preincubated with native unmodifed vimentin peptide. In each western blot, the following antigens were applied to the SDS-PAGE separation: lane 1: human recombinant vimentin, lane 2 = citrullinated human recombinant vimentin, lane 3: carbamylated human recombinant vimentin, lane 4 = acetylated vimentin-albumin conjugate. All incubations were at 10 µg/ for 2 h prior to the immunoblotting experiment.



Supplementary Table 1. Distribution of diagnosis in each outcome group.

Persistent non RA (n=55)	**Resolving arthritis (n=112)**
Unclassified inflammatory arthritis (28)	Unclassified inflammatory arthritis (73)
Psoriatic arthritis (11)	Reactive arthritis (18)
Reactive arthritis (4)	Crystal arthritis (11)
SLE related arthritis (4)	Psoriatic arthritis (6)
IBD related arthritis (3)	Sarcoid related arthritis (3)
Primary Sjogren's syndrome related arthritis (2)	IBD related arthritis (1)
Sarcoid related arthritis (2)	
Polymyalgia rheumatica (1)	

RA: rheumatoid arthritis; IBD: inflammatory bowel disease; SLE: systemic lupus erythematosus. In brackets: number of patients with each diagnosis.

Supplementary Table 2. Sensitivity, specificity, positive predictive value and negative predictive value of antibody isotypes when comparing RA patients vs. persistent non-RA arthritis patients of disease >3 months duration at sample collection.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Anti-CCP	73.0% (55.9-86.2)	96.9% (79.6-99.3)	96.4% (81.6-99.4)	70.6% (52.5-84.9)
Rheum Factor				
IgG	75.7% (58.8-88.2)	76.0% (54.9-90.6)	82.4% (65.5-93.2)	67.9% (47.7-84.1)
IgA	64.9% (47.5-79.8)	80.0% (59.3-93.1)	82.8% (64.2-94.1)	60.6% (42.2-77.1)
Anti-CitVimentin				
IgG	64.9% (47.5-79.8)	72.0% (50.6-87.9)	77.4% (58.9-90.4)	58.1% (39.1-75.4)
IgA	59.5% (42.1-75.2)	76.0% (54.9-90.6)	78.6% (59.0-91.7)	55.9% (37.9-72.8)
Anti-CarVimentin				
IgG	32.4% (18.0-49.8)	100% (86.2-100)	100% (73.4-100)	50.0% (35.5-64.5)
IgA	32.4% (18.0-49.8)	88.0 CI 68.8-97.3	80.0% (51.9-95.4)	80.0% (51.9-95.4)
Anti-AcVimentin				
IgG	24.3% (11.8-41.2)	100% (86.2-100.0)	100% (66.2-100)	47.2% (33.3-61.4)
IgA	10.8% (3.1-25.4)	92.0% (73.9-98.8)	66.7% (22.7-94.7)	41.1% (28.1-55.0)

PPV: positive predictive value, NPV: negative predictive value, CI: confidence interval

Supplementary Table 3. Demographic, clinical and laboratory characteristics of patients in each outcome group. Data are shown as number (percentage), mean ± standard deviation or median (interquartile range) as appropriate. Patients classified according to 2010 ACR EULAR classification criteria for RA.

	Anti-CCP positive RA (n=49)	**Anti-CCP negative RA (n=54)**	**Persistent non RA (n=67)**	**Resolving arthritis (n=98)**	**p value**
Gender, n (%)					
Female	31 (63.3)	28 (51.9)	38 (56.7)	47 (48)	0.331
Age (yrs)	55.4±14.3	56.3±16.2	51.9±18.9	45.7±16.7	<0.001
Disease duration (days)	54.9±21.7	52.2±21.2	55.9±21.6	45.1±20.7	0.005
CRP (mg/dl)	18 (6.3-43.8)	10 (0-39)	17 (8-35)	8 (0-17)	0.001

ESR (mm/hr)	28 (18-51.3)	18 (12-45)	25 (14-53)	14 (6-27)	<0.001
DAS28 (CRP)	4.4±1.4	4.4±1.4	3.7±1.0	2.9±1.2	<0.001
DAS28 (ESR)	4.8±1.5	4.7±1.4	4.0±1.2	3.1±1.4	<0.001
Smoking, n (%)					0.05
Ever-smoker	28/48 (58.3)	29/50 (58)	27/65 (41.5)	36/91 (39.6)	
Never-smoker	20/48 (41.7)	21/50 (42)	38/65 (58.5)	55/91 (60.4)	
Anti-CCP Pos., n (%)	49 (100)	0 (0)	1 (1.5)	1 (1)	<0.001
RF Pos., n (%)	37/49 (75.5)	6/50 (12)	3/59 (5.1)	5/90 (5.5)	<0.001

RA: rheumatoid arthritis, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, DAS: disease activity score, CCP: cyclic citrullinated peptide, RF: rheumatoid factor.

Supplementary Table 4. 4A,Sensitivity, specificity, positive predictive value and negative predictive value of antibody isotypes when comparing RA patients vs. non-RA patients (persistent non-RA and resolving). Patients classified according to 2010 ACR EULAR classification criteria for RA.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Anti-CCP	47.6% (37.6-57.7)	98.8% (95.7-99.8)	96.1% (86.5-99.4)	75.1% (68.8-80.7)
Rheum Factor				
IgG	52.4% (42.4-62.4)	90.9% (85.8-94.8)	78.2% (66.7-87.3)	75.4% (68.8-81.2)
IgA	33.0% (24.2-43.0)	90.9% (85.5-94.8)	69.4% (54.6-81.7)	68.5% (61.9-74.6)
Anti-CitVimentin				
IgG	55.3% (45.2-65.1)	83.0% (76.4-88.4)	67.1% (56.0-76.9)	74.9% (67.9-80.9)
IgA	42.7% (33.0-52.9)	72.7% (65.3-79.4)	49.4% (38.7-60.3)	67.0% (59.6-73.9)
Anti-CarVimentin				
IgG	34.0% (25.0-44.0)	93.9% (89.1-97.1)	77.8% (62.9-88.8)	69.5% (63.0-75.5)
IgA	12.6% (6.9-20.6)	85.5% (79.1-90.5)	35.1% (20.2-52.5)	61.0% (54.4-67.4)
Anti-AcVimentin				
IgG	35.9% (26.7-46.0)	86.1% (79.8-91.0)	61.7% (48.2-73.9)	68.3% (61.5-74.5)
IgA	33.0% (24.1-43.0)	63.0% (55.2-70.4)	35.8% (26.2-46.3)	60.1% (52.4-67.5)

PPV: positive predictive value, NPV: negative predictive value, CI: confidence interval

4B, Sensitivity, specificity, positive predictive value and negative predictive value of antibody isotypes when comparing CCP positive RA patients vs. non-RA patients (persistent non-RA and resolving). Patients classified according to 2010 ACR EULAR classification criteria for RA.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Anti-CCP	100% (92.7-100)	98.8% (95.7-99.8)	96.1% (86.5-99.4)	100% (97.7-100)
Rheum Factor				
IgG	91.8% (80.4-97.7)	90.9% (85.5-94.8)	75.0% (62.1-85.3)	97.4% (93.5-99.3)
IgA	55.1% (40.2-69.3)	90.9% (85.5-94.8)	64.3% (48.0-78.4)	87.2% (81.3-91.8)
Anti-CitVimentin				
IgG	93.9% (83.2-98.6)	83.0% (76.4-88.4)	62.2% (50.1-73.2)	97.9% (93.9-99.5)
IgA	63.3% (48.3-76.6)	72.7% (65.3-79.4)	40.8% (29.7-52.7)	87.0% (80.2-92.1)
Anti-CarVimentin				

IgG	67.4% (52.5-80.0)	93.9% (89.1-97.1)	76.7% (61.4-88.2)	90.6% (85.3-94.6)
IgA	16.3% (7.3-29.7)	85.5% (79.1-90.5)	25% (11.5-43.3)	77.5% (70.7-83.3)
Anti-AcVimentin				
IgG	61.2% (46.2-74.8)	86.1% (79.8-91.0)	56.6% (42.3-70.2)	88.2% (82.2-92.7)
IgA	40.8% (27.0-55.8)	63.0% (55.1-70.4)	24.7% (15.8-35.5)	78.2% (70.2-84.9)

PPV: positive predictive value, NPV: negative predictive value, CI: confidence interval

4C, Sensitivity, specificity, positive predictive value and negative predictive value of antibody isotypes when comparing CCP negative RA patients vs. non-RA patients (persistent non-RA and resolving). Patients classified according to 2010 ACR EULAR classification criteria for RA.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Anti-CCP	0.0% (0.0-6.7)	98.8% (95.7-99.8)	0.0% (0.0-84.2)	75.1% (68.8-80.7)
Rheum Factor				
IgG	16.7% (8.0-29.3)	90.9% (85.5-94.8)	37.5% (18.8-59.4)	76.9% (70.4-82.6)
IgA	13.0% (5.4-24.9)	90.9% (85.5-94.8)	31.8% (13.9-54.9)	76.1% (69.6-81.9)
Anti-CitVimentin				
IgG	20.4% (10.7-33.5)	83.0% (76.4-88.4)	28.2% (15.0-44.9)	76.1% (69.2-82.1)
IgA	24.1% (13.5-37.6)	72.7% (65.3-79.4)	22.4% (12.5-35.3)	74.5% (67.1-81.1)
Anti-CarVimentin				
IgG	3.7% (0.6-12.8)	93.9% (89.1-97.1)	16.7% (2.6-48.4)	74.9% (68.4-80.6)
IgA	9.3% (3.1-20.3)	85.5% (79.1-90.5)	17.2% (5.9-35.8)	74.2% (67.4-80.3)
Anti-AcVimentin				
IgG	13.0% (5.4-24.9)	86.1% (79.8-90.9)	23.3% (10.0-42.3)	75.1% (68.3-81.1)
IgA	26.0% (15.0-39.7)	63.0% (55.2-70.4)	18.7% (10.6-29.3)	72.2% (64.2-79.4)

PPV: positive predictive value, NPV: negative predictive value, CI: confidence interval