The combination of three autoantibodies, ACPA, RF and anti-CarP antibodies is highly specific for rheumatoid arthritis: *implications for very early identification of individuals at risk to develop rheumatoid arthritis.*

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Conflict of interest
Leendert Trouw, Tom Huizinga and Rene Toes are listed as inventors on a patent on the detection of anti-CarP antibodies in RA

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Abstract

Objective In rheumatoid arthritis(RA), the autoantibodies anti-citrullinated protein antibodies(ACPA) and rheumatoid factor(RF) are commonly used to aid RA diagnosis. Although these autoantibodies are mainly found in RA, their specificity is not optimal. It is therefore difficult to identify RA patients, especially in very early disease, based on the presence of ACPA and RF alone. Also, anti-carbamylated protein(anti-CarP) antibodies have diagnostic and prognostic value as the presence of anti-CarP antibodies associates with joint damage in RA patients and with future RA development in arthralgia patients. Therefore, we aimed to investigate the value of combined antibody testing in relation to prediction and diagnosis of (early) RA.

Methods A literature search resulted in twelve studies, consisting of RA patients, pre-RA individuals, disease controls, healthy first-degree relatives of RA patients or healthy controls, in which data on RF, ACPA and anti-CarP antibody-status was available. Random effects meta-analyses were carried out for several antibody combinations.

Results The individual antibodies are highly prevalent in RA(34%-80%) compared to the control groups, but are also present in non-RA controls(0%-23%). To classify most people correctly as RA or non-RA, the combination of ACPA and/or RF often performs well(specificity:65-100, sensitivity:59-88). However, triple positivity for ACPA, RF and anti-CarP antibodies results in a higher specificity(98-100) (accompanied by a lower sensitivity(27-39).

Conclusions As the rheumatology field is moving towards very early identification of RA and possible screening for individuals at maximum risk in populations with a low pre-test probability, triple positivity provides interesting information on individuals at risk to develop RA.

Keywords: Rheumatoid arthritis, autoantibodies, anti-carbamylated protein antibodies, rheumatoid factor, anti-citrullinated protein antibodies

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease, characterized by immune cell infiltration in the joint, joint pain and possibly cartilage and bone degradation. In RA, several antibody systems have been identified based on their target antigens. Two of these autoantibodies, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), have also been incorporated into the classification criteria for RA(1). RFs are antibodies that recognize the Fc tail of other (IgG) antibodies, while ACPA recognize proteins that contain citrulline(s), which arise by a post-translational modification. While ACPA and RF are highly prevalent in RA they can also be identified in a small percentage of healthy controls(2-4). In a meta-analysis, comparing RA patients to healthy controls for the presence of ACPA (measured by CCP in this meta-analysis (cyclic citrullinated peptide)), a pooled sensitivity of 67% was observed, while this was 69% for IgM-RF. The combined specificity was 95% for ACPA and 85% for IgM-RF(5). Although more than half of the RA patients are positive for ACPA and/or RF, a substantial part of the patients cannot be identified in this manner. To date, it is unclear whether it will be possible to fill this serological gap(6) with other (antibody) biomarkers.

Importantly, ACPA and RF can both be detected more than 10 years before disease onset(7), which would possibly allow for early identification of individuals at risk to develop RA. However, less than 50% of the ACPA positive patients with non-specific musculoskeletal symptoms develop RA after 1 year(8). Also, less than 50% of the ACPA- and RF-double-positive arthralgia patients develop RA after up to 2 years of follow-up(9). The presence of ACPA and/or RF is therefore not sufficient for the prediction of RA development. In RA patients, there seems to be a “window of opportunity” in the early phase of disease. Treatment during this phase may increase the amount of RA patients that reach drug-free remission, effectively reducing the number of individuals with chronic disease(10). However, since treatment of asymptomatic individuals may not be free from side effects, it is

important to identify the individuals at risk to develop RA as accurately as possible and minimize misclassification and unnecessary side effects of treatments.

Besides ACPA and RF, several other autoantibodies, such as anti-CarP antibodies, anti-PAD antibodies and anti-malondialdehyde antibodies have been identified in RA patients(11, 12). Of these autoantibodies, antibodies that target carbamylated proteins (anti-CarP antibodies) have been studied extensively(13). Carbamylation is a post-translational modification, which can arise via a chemical reaction with cyanate, converting a lysine into a homocitrulline. Anti-CarP antibodies can also be present before disease onset(14-17) and have been measured and analysed in a substantial number of RA patients(13-36) and other conditions(28, 29, 33-35, 37-41). Importantly, anti-CarP autoantibodies also occur in RA patients that are seronegative for both ACPA and RF and may therefore represent an interesting additional biomarker to aid diagnosis of RA patients(13, 25). Here, we have two aims in relation to ACPA, RF and ant-CarP antibody measurement. First, we aimed to determine whether the combination of these three autoantibodies may assist in improving the diagnosis of RA. Second, we investigated whether this autoantibody combination would provide additive value for the prediction of RA development. To investigate this, we combined newly obtained data from several unique cohorts with a literature search to investigate the value of combining anti-CarP antibodies with ACPA and RF. In this meta-analysis of 12 different studies involving over 5000 unique individuals, we show that the presence of ACPA and/or RF, as often used in the clinical setting, seems to perform well to identify diagnosed RA patients; however, the highest specificity for RA is achieved when the three autoantibodies are present at the same time.

Materials and Methods

Study selection and inclusion

PubMed was searched for “anti-CarP antibodies” (and anti-carbamylated protein antibodies). Furthermore, a combined search for “carbamylation” and “antibody” was carried out to identify possible missing studies. A complete overview of the search strategy can be seen in the supplementary data. An additional search in web of science did not result in any additional articles fulfilling the inclusion criteria. Studies were selected based on the following criteria: First, antibody data had to be available on ACPA, RF and anti-CarP antibodies for at least two groups, such as RA patients and controls or RA patients and healthy first-degree relatives (HFDR). Studies describing these antibodies in non-RA patients without a comparison to RA were excluded. Second, since the assay to measure anti-CarP antibodies is not yet commercially available, similar antigens, in this case carbamylated fetal calf serum (Ca-FCS), had to be used for the measurement of anti-CarP antibodies. Third, the controls that were included had to be geographically matched controls. The following subgroups were included: RA patients, HFDR, pre-RA and healthy controls.
After the selection, data were extracted from the papers with a standard form, describing the number of patients positive for each of the possible antibody combinations. If data could not be acquired from the published papers, authors were approached for further information.

Data analysis

Informative antibody combinations (Anti-CarP alone, ACPA alone, RF alone, RF and/or ACPA, RF and ACPA, RF and/or ACPA and anti-CarP, at least 1 antibody, at least 2 antibodies, all 3 antibodies), were selected and used for further analysis. Within each group, the percentage of individuals positive for each antibody combination was calculated. Also, specificity, sensitivity, odds ratios(OR), positive likelihood ratios(LR+) and negative likelihood ratios(LR-) were determined. Calculations were carried out in Microsoft Excel version 2010, SPSS statistics version 23(IBM) or R version 3.2.3(42). The control group did not contain antibody-positive individuals for some antibody combinations, which

interferes with the calculation of ORs and LR+s. To estimate these values, a pseudo-frequency modification was used(43). This modification entails adding a small number to each cell in the contingency table. This number was different for each study and based on the percentage of positives for a certain antibody combination in all of the relevant control samples combined. The replacement values added varied between 0.04 and 1.

Meta-analyses were carried out in Stata version 14 using an inverse variance random effects model, resulting in combined ORs as output. The meta-analysis was carried out for the selected antibody combinations, separately for each of the categories(RA vs Healthy controls; RA vs HFDR; RA vs disease controls; Pre-RA vs No RA development). For the RA vs disease controls group, two studies(35, 41) were combined before the meta-analysis, since the RA population in both studies was the same.

Results

Study inclusion and exclusion

A total of 12 publications were included in the analysis. Table 1 shows an overview of the included studies and the number of patients included in each of the different groups. The studies that were excluded either investigated less than two groups, making it impossible to compare groups, or none of the groups included were RA patients(32, 37-40). Studies were also excluded because data on the control group was not available for one or more of the three antibodies(18, 19, 25, 36) or because persons negative for ACPA or RF were excluded from the study(17). Furthermore, some studies used a different antigen than Ca-FCS to measure anti-CarP antibodies(33, 44, 45) and a study did not use geographically matched controls(24). Finally, the IMPROVED study was excluded since part of the patients overlap with the patients in the Leiden EAC study which was included(13, 20). All twelve of the studies included were retrospective studies using a case-control setting. However, three studies were nested case-control studies, all investigating serum samples of RA patients before RA development(14-16). Although prospective studies would have been ideal to include in our study, the only prospective study available had to be excluded due to patient / control group selection based on antibody status(17).

In all of the studies that were included, the ACR 1987 criteria were used for the diagnosis of RA patients. Furthermore, ACPA were measured with anti-CCP2 in all studies, except one(26), in which positivity for CCP2 or CCP3 was used. For RF measurement, RF-IgM was measured in each of the included cohorts.

Prevalence of ACPA, RF and anti-CarP antibodies

To acquire more insight into the data that was acquired, initially, simple overviews of the data were made. The different studies could be divided into 4 subgroups, namely RA patients compared to healthy controls, RA patients compared to disease controls, RA patients compared to HFDR and RA

patients before disease development (pre-RA) compared to healthy controls (Figure 1A). ACPA, RF and anti-CarP antibody positivity were compared between the different studies within each category (Figure 1B-D). Within, for example RA patients, ACPA-positivity was 50%-78%, RF-positivity 53%-80% and anti-CarP-positivity 34%-53%. This indicates that within each subgroup, there is some variation with regards to antibody positivity. However, the most obvious differences are between the 4 subgroups, indicating that these subgroups should not be combined in a meta-analysis.

General presence of autoantibody combinations

Since we hypothesize that the combination of three autoantibodies may provide additional insight in diagnosis or prediction of RA, we set out to investigate different autoantibody combinations that may co-occur within one individual. The number of autoantibodies, (0, 1, 2 or 3) present in the samples in the different studies is shown in Figure 2A-D. In the RA patient studies, we observed that a large proportion of the patients is positive for at least one antibody, but also the combination of two and especially three antibodies is common in RA patients (mean number of autoantibodies between 1.4 and 2.1). For the other groups, it was most common to observe positivity for none of the antibodies. However, positivity for one of the three antibodies or a combination of multiple antibodies was not completely absent. The lowest number of antibodies was observed in healthy controls (mean between 0.0 and 0.2) while a larger number could be detected for HFDR (mean 0.4) and disease controls (mean between 0.2 and 0.4).

These data indicate that a large proportion of RA patients has at least one antibody subgroup and more than 40% of the RA patients can be positive for two or three of these antibodies. This pattern is completely different in healthy controls, in which the presence of 1 or 2 antibodies can be observed only in a limited number of people, while the presence of all three of the autoantibodies at the same time is absent in nearly all healthy controls. The other groups, pre-RA, HFDR and disease controls have a slightly higher number of autoantibodies than the healthy controls, but less than the RA patients. The combination of three autoantibodies is also rare in these control groups. Because

the combination of three autoantibodies is rare in the control groups, this may be the most interesting antibody combination for further investigation, although some of the other antibody combinations may show surprising results as well. A complete overview of the different autoantibody combinations in the studies are shown in Venn-diagrams in Figure 3.

Sensitivity, Specificity, Odds ratio, LR+ and LR-

To further investigate these observations, several antibody combinations were studied with a focus on the following four: ACPA and/or RF, ACPA and/or RF and anti-CarP antibodies, two out of the three antibodies and three out of the three antibodies. ACPA and/or RF was chosen since this is what has been incorporated into the current guidelines for RA classification(1). The second combination adds anti-CarP antibodies to the current standard. We hypothesize that the presence of all three autoantibodies at the same time would be the most specific for the diagnosis or prediction of RA. Therefore we also included this combination and as a second option investigated whether the presence of two different autoantibodies out of the three investigated would also result in increased specificity. In general, an increase in the number of antibodies results in a higher specificity and OR, while decreasing the sensitivity. For example, for RA patients compared to healthy controls, the specificity for 1 antibody varies between 85.7 and 97.2, while this is between 98.7 and 100 for the combination of three antibodies. However, the sensitivity for 1 antibody in this same group is between 60.5 and 90.2 and between 30.8 and 39.1 for the combination of three autoantibodies. Interestingly, in many of the studies, a 100% specificity can be achieved with certain antibody combinations. This occurs most frequently for the combination of all three autoantibodies. This indicates that, in the case-control settings studied, the subjects without RA could be identified perfectly by the absence of the combination of the three autoantibodies.. An overview of the specificity, sensitivity, OR, for several antibody combinations is shown in table 2. An overview for other, selected, antibody combinations is shown in supplementary table 1. An overview of the LR+s and LR-s for the same antibody combinations can be seen in supplementary table 2. An complete

overview of the number of antibody-positive people for each group and the ORs can be seen in supplementary table 3.

Meta-analysis

A random effect meta-analysis was carried out on the ORs calculated for each of the discussed antibody combinations. These calculations were carried out separately for each of the different categories, since the differences between these categories are too large to combine the data. An overview of the meta-analysis can be found in the supplementary figures, and a summary is provided in Figure 4A-D. When we are interested in the diagnosis of RA, the two most interesting subgroups would be the Ra patients compared to disease controls or healthy relatives.
When comparing RA to healthy controls, disease controls or healthy relatives, the combination of ACPA and/or RF seems to perform very well, and might be rather similar to the combination of all three autoantibodies. This indicates that the autoantibodies that are currently in use for the diagnosis of RA be sufficient and not much improvement may be gained upon the addition of anti-CarP antibodies. When we are interested in the prediction of RA, it is most important to compare the group in which antibodies were measured before RA development and compared to people without RA. Here a clear increase in OR, with an OR over 100, can be observed for 3 autoantibodies when compared to all of the other combinations. This indicates that, especially in a setting of very early RA, the presence of 3 antibodies results in the highest odds for developing RA. Therefore, this combination may help in predicting the development of RA.

Discussion

Here we aimed to investigate the additional value of anti-CarP antibodies compared to ACPA and RF in two different settings, diagnosis and prediction. Therefore, we carried out a literature search and described the studies in which RF, ACPA and anti-CarP antibodies were measured. In a meta-analysis we eventually conclude that measuring all three of these antibodies reduces the chance to misclassify non-RA controls, but may not improve the diagnosis of RA. Therefore the analysis for triple positivity may be especially relevant for populations with a low pre-test probability, although sensitivity will be low with these measurements. These findings are of relevance in view of the efforts towards pre-emptive treatments for people at risk.

A previous meta-analysis has investigated anti-CarP antibodies in RA patients compared to healthy controls(46), resulting in a pooled OR of 17 for anti-CarP antibodies alone. Our OR derived from the meta-analysis for anti-CarP antibodies alone when comparing RA patients to healthy controls was 30, which is slightly different, possibly because there were differences in inclusion criteria. The previous study however, did not compare any antibody combinations within the same patient groups and only investigated RA patients compared to healthy controls. In our study, we also compared RA patients to disease controls and HFDR, which are known to have higher autoantibody positivity than healthy controls. The comparison to disease controls is especially important, since the studied antibodies can also be present in non-RA populations(41, 47). Furthermore, we also investigated the number of autoantibodies present in people before RA development(pre-RA) and compared these to healthy controls. One of the studies published after we selected the articles for our meta-analysis, confirms our observations that the use of ACPA and RF might be sufficient after RA diagnosis, although also in this case the addition of anti-CarP antibodies increases the specificity, in exchange for a reduced sensitivity(48).

One of the limitations of this study is that all of the cohorts included were case-control or nested case controls studies and not prospective cohorts. Unfortunately, none of the prospective cohorts available fulfilled the inclusion criteria. Another limitation of this study might be that the assay for anti-CarP antibody measurement is not yet a commercially available, indicating that there might be differences in these measurements. Therefore, rather strict criteria were made with regards to study inclusion, thereby eliminating some interesting studies that could not be included. Also, some of the studies may have used different methods to determine the cut-off of their assay, however we have used the original data on antibody positivity as described in each individual article. An analysis of antibody levels with regards to RA development may also be interesting, but insufficient data was available for such an analysis. Furthermore, we do not have data on the stability of the three biomarkers in these patients and whether any seroconversion may occur over time in the patients analysed.

Out of several antibody combinations, measuring all three autoantibodies, ACPA, RF and anti-CarP antibodies or measuring ACPA and RF, often results in the highest specificity and LR+, thereby reducing the sensitivity. Therefore, depending on the context of the investigation, one of the antibody combinations might be more suitable than the other. When aiming to identify RA patients as early as possible, the most relevant group to study would be the group including people before RA, which are currently present in the healthy population. Interestingly, in this group, there is a clearly higher OR for the combination of all three autoantibodies, suggesting that the combination of anti-CarP antibodies, ACPA and RF might result in an improvement of the early identification of people at risk to develop RA.

While the antibody-based biomarkers provide an interesting and robust method to identify persons at risk to develop RA, this will, in the current setting, identify less than 50% of the (future) RA patients as the others are negative for these biomarkers(8, 9) . Whether the identification of

additional biomarkers will close this “serological gap” remains to be seen(6). Other biomarkers or early clinical symptoms may serve as additional input into the risk stratification. Furthermore, it has been suggested that the early identification of RA patients, or the identification of arthralgia patients that are at high risk of developing RA is important for effective treatment of RA(49-51). The combination of these three autoantibodies may help in to identify these high-risk patients. Attractive in the three-autoantibody-approach highlighted here is the low-cost of the assays and equipment, the nature of the sample to be used (serum) and the stability of the antibodies in serum. Moreover, the ease of testing and interpretation of these tests allows for feasible implementation for large-scale testing to identify patients at risk for RA in contrast to other proposed methodologies such as imaging-based tests.

In order to further investigate whether this would be a suitable option and whether the addition of anti-CarP antibodies will result in an increased detection of people at risk for the development of RA, carrying out prospective studies in large healthy populations would be appropriate. In conclusion, the combination of anti-CarP antibodies, ACPA and RF has a very high specificity for the identification of early RA patients compared to different types of controls.

Table 1 – An overview of the number of people present in each of the included studies, separated for each category.

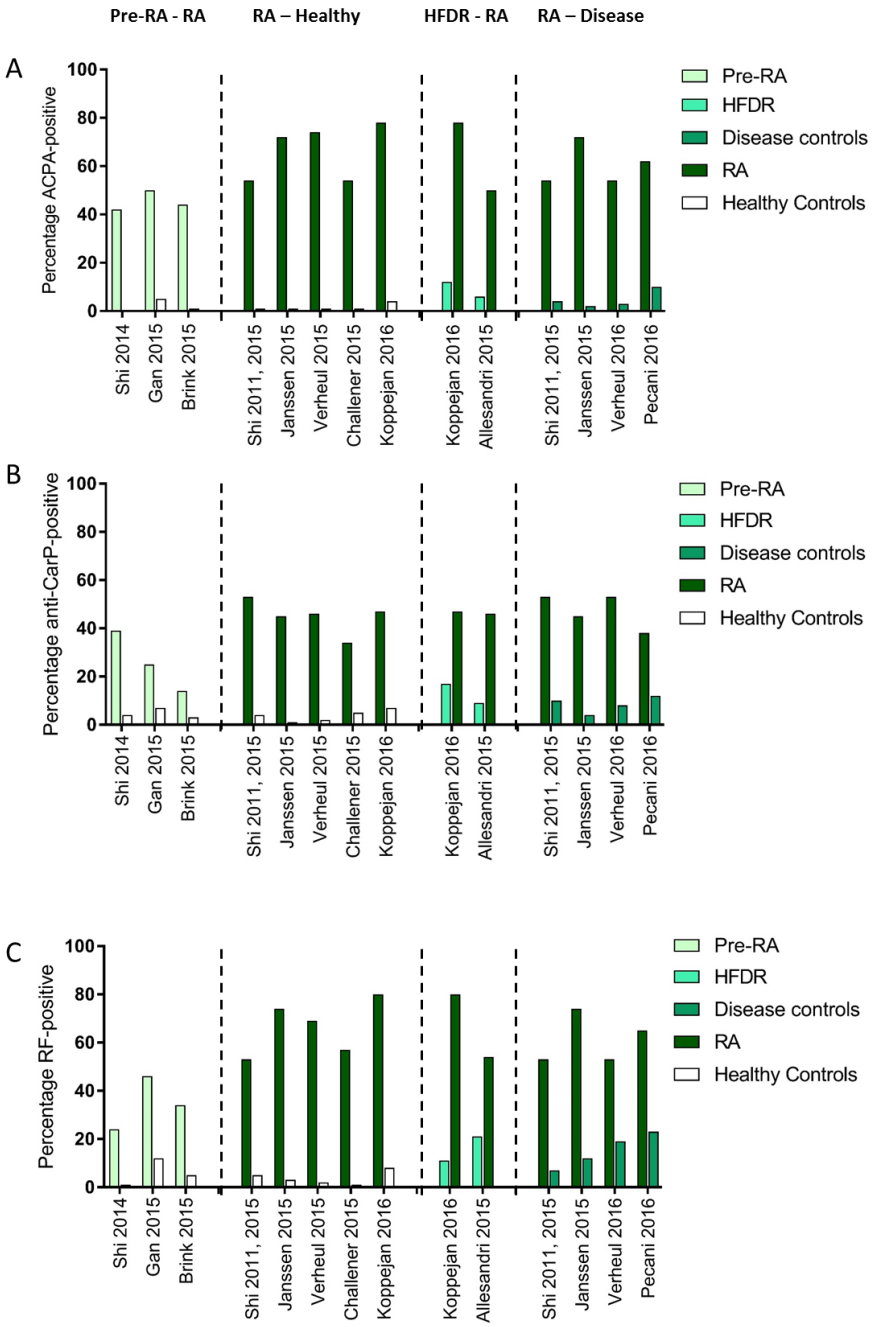
Cohort	RA development	First-degree relatives	Disease controls	RA	Healthy Controls	References
Shi 2011, Shi 2015, the Netherlands			780	934	208	(13, 41)
Janssen 2015, the Netherlands			235	86	36	(34)
Verheul 2015, Japan				268	127	(52)
Challener 2015, Canada / USA				517	63	(23)
Koppejan 2016, Canada		105		92	77	(26)
Allesandri 2015, Italy		141		63		(21)
Verheul 2016, the Netherlands			759	934		(35)
Pecani 2016, Italy			298	309		(29)
Shi 2014 The Netherlands	79				141	(16)
Gan 2015 USA	76				41	(14)
Brink 2015 Sweden	224				150	(15)

RA; rheumatoid arthritis, HFDR; healthy first-degree relatives. Shi 2011 and Shi 2015 make use of the same RA patient cohort as a comparison to either healthy controls or disease controls. The same RA-population was also shown as a comparison for the Verheul 2016 publication.

		ACPA and / or RF			ACPA / and or RF and anti-CarP			2 antibodies			3 antibodies		
		Spec	Sens	AUC	Spec	Senc	AUC	Spec	Sens	AUC	Spec	Sens	AUC
RA vs Healthy	Shi 2011	94.2	67.5	0.808	100	48.6	0.743	99.5	52.7	0.761	100	35	0.675
	Janssen 2015	97.2	87.2	0.922	100	44.2	0.721	100	68.6	0.843	100	34.9	0.674
	Verheul 2015	97.6	80.2	0.889	99.2	44	0.716	99.2	69	0.841	100	37.3	0.687
	Challener 2016	100	59	0.795	100	32.5	0.662	100	53.4	0.767	100	30.8	0.654
	Koppejan 2016	90.9	88	0.895	98.7	44.6	0.716	97.4	76.1	0.867	98.7	39.1	0.689
RA vs first-degree relatives	Koppejan 2016	80	88	0.84	92.4	44.6	0.685	90.5	76.1	0.833	99	39.1	0.691
	Alessandri 2016	74.5	65.1	0.698	95	30.2	0.626	93.6	49.2	0.714	99.3	19	0.592
RA vs Disease controls	Janssen 2015	87.2	87.2	0.872	98.7	44.2	0.715	97.9	68.6	0.832	99.6	34.9	0.672
	Shi 2015	90.5	67.5	0.79	97.6	48.6	0.731	96.7	52.7	0.747	99.1	35	0.671
	Verheul 2016	78.8	67.5	0.731	97.4	48.6	0.73	97.2	52.7	0.75	99.6	35	0.673
	Pecani 2016	73.2	71.5	0.723	92.6	29.4	0.61	88.9	58.3	0.736	98	26.5	0.623
Before RA vs No RA	Shi 2014	100	44.3	0.722	100	32.9	0.665	100	34.2	0.671	100	20.3	0.601
	Gan 2015	85.4	60.5	0.729	97.6	23.7	0.606	95.1	42.1	0.686	100	17.1	0.586
	Brink 2015	93.3	50	0.717	100	12.9	0.565	100	30.4	0.652	100	10.7	0.554

Table 2 – Sensitivity, specificity and AUCs are shown for 4 different antibody combinations

RA; rheumatoid arthritis, HFDR; healthy first-degree relatives, spec; specificity, sens; sensitivity, OR; odds ratio, ACPA; anti-citrullinated protein antibodies, RF; rheumatoid factor, anti-CarP; anti-Carbamylated protein antibodies, AUC; area under the curve. The sensitivity, specificity and AUC for 5 more antibody combinations (namely, ACPA only, anti-CarP only, RF only, at least 1 antibody and RF and ACPA are shown in supplementary table 1



Figures

Figure 1 –ACPA, RF and anti-CarP antibody status are similar in studies fulfilling the inclusion criteria.

Studies were separated based on the category they were placed in (RA patients compared to healthy controls; People who developed RA after a certain timespan compared to people who do not develop disease; RA patients compared to healthy first-degree relatives and RA patients compared to disease controls). Some studies occur twice, as they fit more than one category. The percentage of antibody-positive people for each of these studies are shown in A) for ACPA, B) for anti-CarP antibodies and C) for RF. The number of patients in each study can be found in table 1. RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, Anti-CarP; anti-carbamylated protein antibodies, RF; rheumatoid factor.

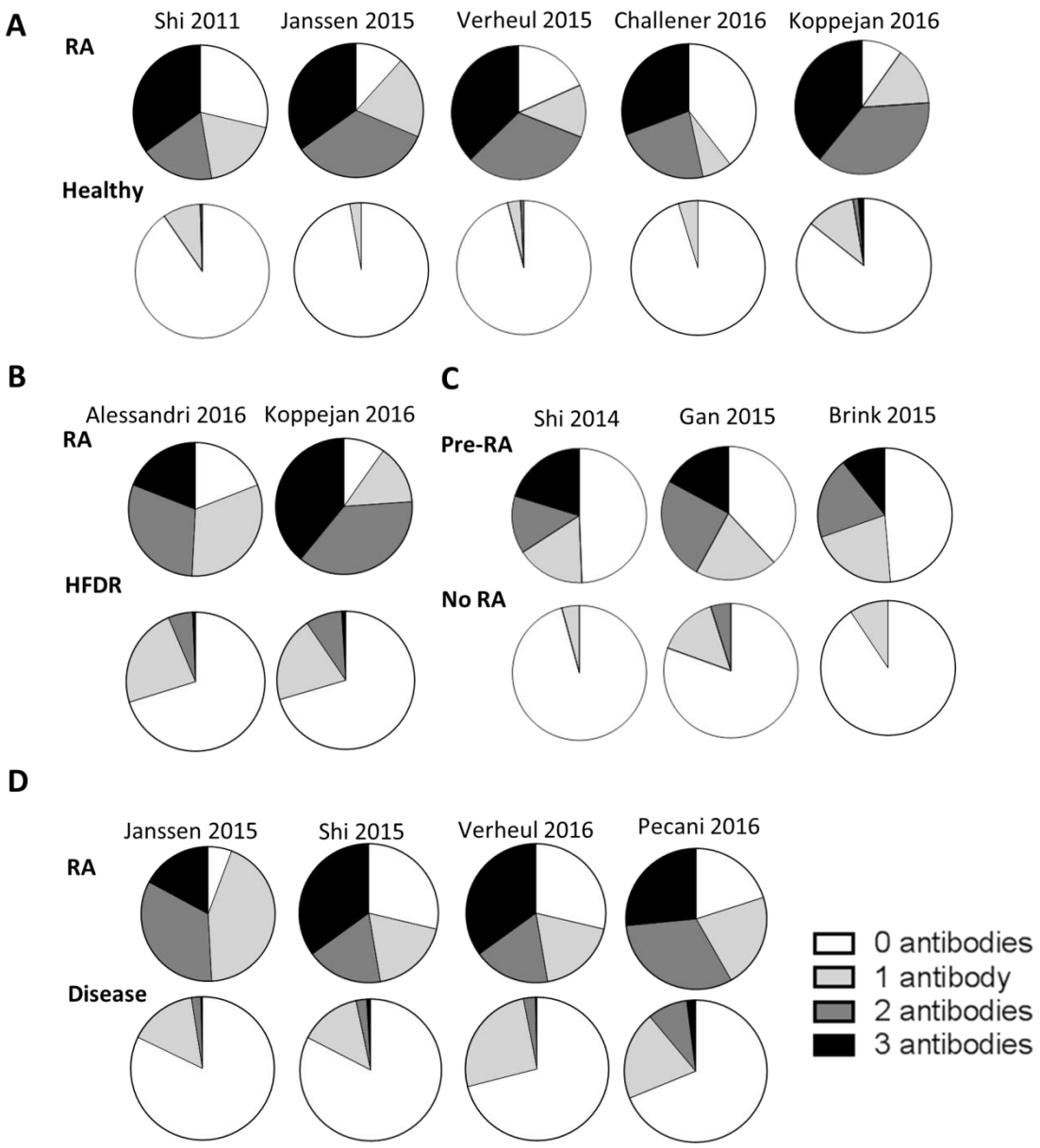


Figure 2 – The number of antibodies is increased in RA patients when compared to non-RA controls.

The number of antibodies is show in pie charts for each of the included studies, showing the comparison for RA vs healthy controls (A), RA vs healthy first-degree relatives (HFDR) (B), Before RA development (Pre-RA)and no RA development (C) and RA vs disease controls (D). For each of the figure, the upper part shows the patients with or who will develop RA, while the lower part shows the respective control group. The number of patients in each study can be found in table 1. RA; rheumatoid arthritis.

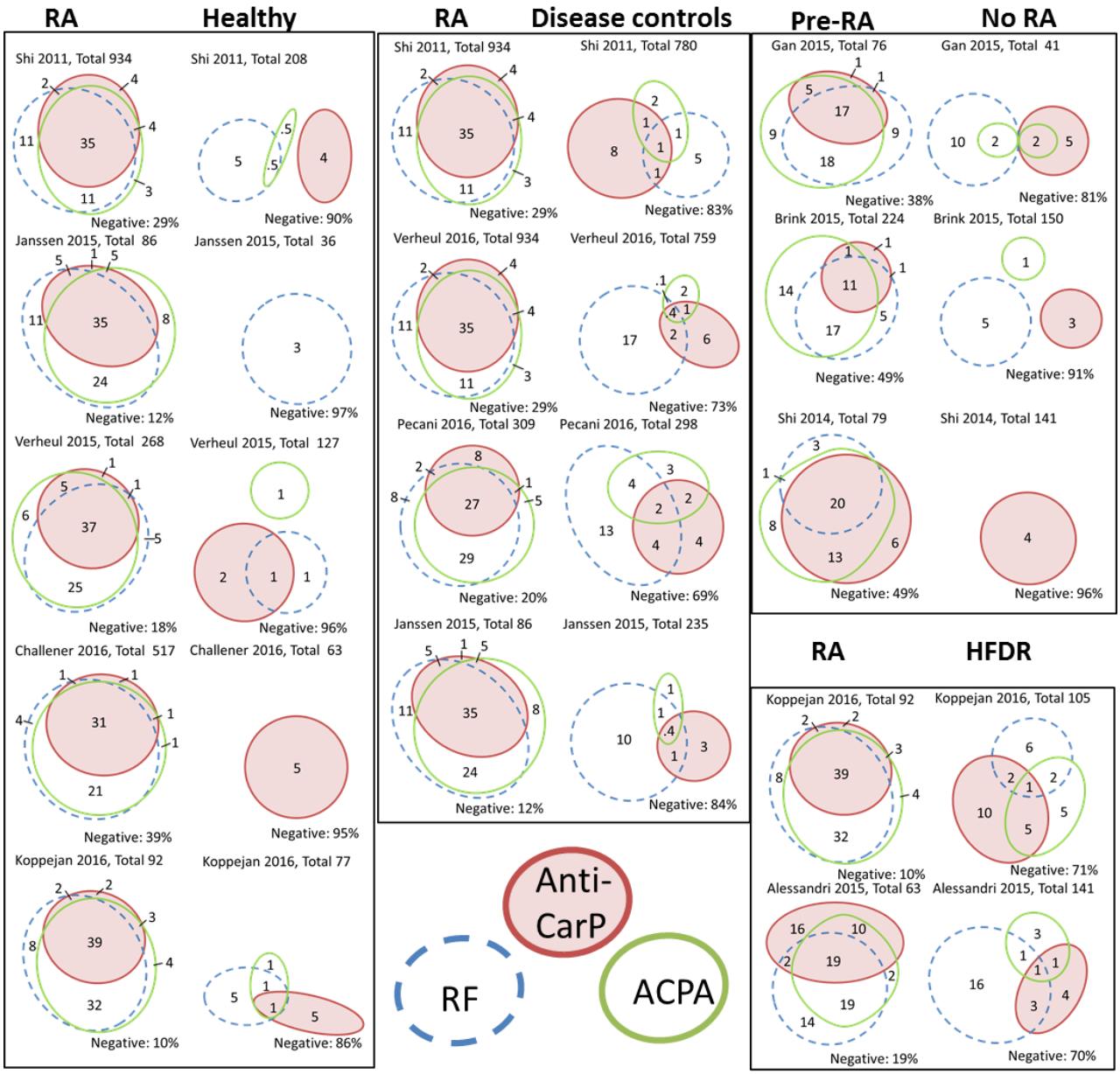


Figure 3 – Detailed overview of autoantibody-status and antibody combinations in the studies included.

A detailed overview of the combinations of positivity for ACPA, RF or anti-CarP antibodies is shown in Venn diagrams for RA vs healthy controls, RA vs disease controls, Pre-RA vs RA and RA vs healthy first-degree relatives. The filled red circle represents anti-CarP antibody-positivity. The dashed blue circle shows the RF-positivity and the green solid circle indicates ACPA-positivity. The size of a circle represents the percentage of people with that specific antibody combination when compared to other antibody positive people in the study. Sizes of the circles cannot be compared between groups and are an approximation of the true percentages. The basis for the Venn-diagrams was made in EulerAPE. RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, anti-CarP; anti-carbamylated protein, RF; rheumatoid factor, HFDR; healthy first-degree relatives.

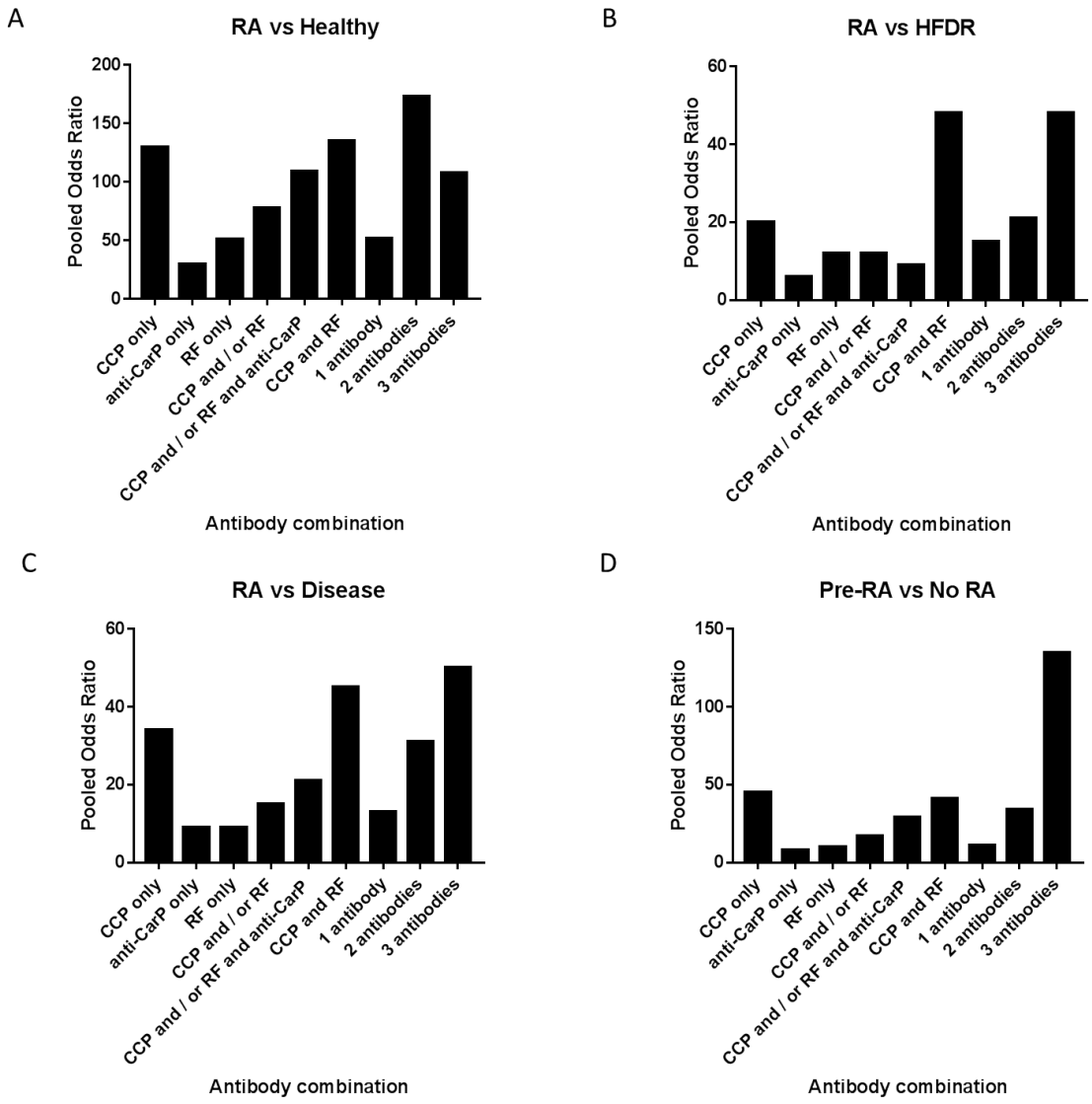


Figure 4 – Overview of odds ratios derived from random effects meta-analyses.
Pooled odds ratios are shown separated for the different categories of patients and controls: RA vs healthy controls (A), RA vs healthy first-degree relatives (HFDR) (B), RA vs disease controls (C) and pre-RA vs no RA (D). Random effects meta-analysis was carried out for each of the studies antibody combinations. For the comparison between RA and disease controls two studies were combined before analysis, since the same RA patient cohort was used for comparison (35, 41). An overview of the meta-analyses, with the individual forest plots, can be seen in the supplementary figures. Also, an overview of the individual numbers of antibody-positive patients and ORs for each cohort can be found in supplementary table 3.

References

1. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis. 2010;69(9):1580-8.
2. van Zanten A, Arends S, Roozendaal C, Limburg PC, Maas F, Trouw LA, et al. Presence of anticitrullinated protein antibodies in a large population-based cohort from the Netherlands. Ann Rheum Dis. 2017;76(7):1184-90.
3. Tasliyurt T, Kisacik B, Kaya SU, Yildirim B, Pehlivan Y, Kutluturk F, et al. The frequency of antibodies against cyclic citrullinated peptides and rheumatoid factor in healthy population: a field study of rheumatoid arthritis from northern Turkey. Rheumatol Int. 2013;33(4):939-42.
4. Terao C, Ohmura K, Ikari K, Kawaguchi T, Takahashi M, Setoh K, et al. Effects of smoking and shared epitope on the production of anti-citrullinated peptide antibody in a Japanese adult population. Arthritis Care Res (Hoboken). 2014;66(12):1818-27.
5. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med. 2007;146(11):797-808.
6. Trouw LA, Mahler M. Closing the serological gap: promising novel biomarkers for the early diagnosis of rheumatoid arthritis. Autoimmun Rev. 2012;12(2):318-22.
7. Brink M, Hansson M, Mathsson-Alm L, Wijayatunga P, Verheul MK, Trouw LA, et al. Rheumatoid factor isotypes in relation to antibodies against citrullinated peptides and carbamylated proteins before the onset of rheumatoid arthritis. Arthritis Res Ther. 2016;18:43.
8. Rakieh C, Nam JL, Hunt L, Hensor EM, Das S, Bissell LA, et al. Predicting the development of clinical arthritis in anti-CCP positive individuals with non-specific musculoskeletal symptoms: a prospective observational cohort study. Ann Rheum Dis. 2015;74(9):1659-66.
9. Bos WH, Wolbink GJ, Boers M, Tijhuis GJ, de Vries N, van der Horst-Bruinsma IE, et al. Arthritis development in patients with arthralgia is strongly associated with anti-citrullinated protein antibody status: a prospective cohort study. Ann Rheum Dis. 2010;69(3):490-4.
10. van Nies JA, Tsonaka R, Gaujoux-Viala C, Fautrel B, van der Helm-van Mil AH. Evaluating relationships between symptom duration and persistence of rheumatoid arthritis: does a window of opportunity exist? Results on the Leiden early arthritis clinic and ESPOIR cohorts. Ann Rheum Dis. 2015;74(5):806-12.
11. Trouw LA, Rispens T, Toes REM. Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. Nat Rev Rheumatol. 2017;13(6):331-9.
12. Verheul MK, Fearon U, Trouw LA, Veale DJ. Biomarkers for rheumatoid and psoriatic arthritis. Clin Immunol. 2015;161(1):2-10.
13. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A. 2011;108(42):17372-7.
14. Gan RW, Trouw LA, Shi J, Toes RE, Huizinga TW, Demoruelle MK, et al. Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. J Rheumatol. 2015;42(4):572-9.
15. Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther. 2015;17:25.
16. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Hamann D, van Schaardenburg D, et al. Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. Ann Rheum Dis. 2014;73(4):780-3.
17. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Toes RE, Trouw LA, et al. Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. Arthritis Rheum. 2013;65(4):911-5.
18. Ajeganova S, Humphreys JH, Verheul MK, van Steenbergen HW, van Nies JA, Hafstrom I, et al. Anticitrullinated protein antibodies and rheumatoid factor are associated with increased mortality but

with different causes of death in patients with rheumatoid arthritis: a longitudinal study in three European cohorts. Ann Rheum Dis. 2016;75(11):1924-32.
19. Ajeganova S, van Steenbergen HW, Verheul MK, Forslind K, Hafstrom I, Toes RE, et al. The association between anti-carbamylated protein (anti-CarP) antibodies and radiographic progression in early rheumatoid arthritis: a study exploring replication and the added value to ACPA and rheumatoid factor. Ann Rheum Dis. 2017;76(1):112-8.
20. Akdemir G, Verheul MK, Heimans L, Wevers-de Boer KV, Goekoop-Ruiterman YP, van Oosterhout M, et al. Predictive factors of radiological progression after 2 years of remission-steered treatment in early arthritis patients: a post hoc analysis of the IMPROVED study. RMD Open. 2016;2(1):e000172.
21. Alessandri C, Bartosiewicz I, Pendolino M, Mancini R, Colasanti T, Pecani A, et al. Anti-carbamylated protein antibodies in unaffected first-degree relatives of rheumatoid arthritis patients: lack of correlation with anti-cyclic citrullinated protein antibodies and rheumatoid factor. Clin Exp Rheumatol. 2015;33(6):824-30.
22. Castaneda-Delgado JE, Bastian-Hernandez Y, Macias-Segura N, Santiago-Algarra D, Castillo-Ortiz JD, Aleman-Navarro AL, et al. Type I Interferon Gene Response Is Increased in Early and Established Rheumatoid Arthritis and Correlates with Autoantibody Production. Front Immunol. 2017;8:285.
23. Challener GJ, Jones JD, Pelzek AJ, Hamilton BJ, Boire G, de Brum-Fernandes AJ, et al. Anti-carbamylated Protein Antibody Levels Correlate with Anti-Sa (Citrullinated Vimentin) Antibody Levels in Rheumatoid Arthritis. J Rheumatol. 2016;43(2):273-81.
24. Humphreys JH, Verheul MK, Barton A, MacGregor AJ, Lunt M, Toes RE, et al. Anticarbamylated protein antibodies are associated with long-term disability and increased disease activity in patients with early inflammatory arthritis: results from the Norfolk Arthritis Register. Ann Rheum Dis. 2016;75(6):1139-44.
25. Jiang X, Trouw LA, van Wesemael TJ, Shi J, Bengtsson C, Kallberg H, et al. Anti-CarP antibodies in two large cohorts of patients with rheumatoid arthritis and their relationship to genetic risk factors, cigarette smoking and other autoantibodies. Ann Rheum Dis. 2014;73(10):1761-8.
26. Koppejan H, Trouw LA, Sokolove J, Lahey LJ, Huizinga TJ, Smolik IA, et al. Role of Anti-Carbamylated Protein Antibodies Compared to Anti-Citrullinated Protein Antibodies in Indigenous North Americans With Rheumatoid Arthritis, Their First-Degree Relatives, and Healthy Controls. Arthritis Rheumatol. 2016;68(9):2090-8.
27. Kumar S, Pangtey G, Gupta R, Rehan HS, Gupta LK. Assessment of anti-CarP antibodies, disease activity and quality of life in rheumatoid arthritis patients on conventional and biological disease-modifying antirheumatic drugs. Reumatologia. 2017;55(1):4-9.
28. Nakabo S, Hashimoto M, Ito S, Furu M, Ito H, Fujii T, et al. Carbamylated albumin is one of the target antigens of anti-carbamylated protein antibodies. Rheumatology (Oxford). 2017.
29. Pecani A, Alessandri C, Spinelli FR, Priori R, Riccieri V, Di Franco M, et al. Prevalence, sensitivity and specificity of antibodies against carbamylated proteins in a monocentric cohort of patients with rheumatoid arthritis and other autoimmune rheumatic diseases. Arthritis Res Ther. 2016;18(1):276.
30. Verheul MK, Yee A, Seaman A, Janssen GM, van Veelen PA, Drijfhout JW, et al. Identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis. J Autoimmun. 2017;80:77-84.
31. Vidal-Bralo L, Perez-Pampin E, Regueiro C, Montes A, Varela R, Boveda MD, et al. Anti-carbamylated protein autoantibodies associated with mortality in Spanish rheumatoid arthritis patients. PLoS One. 2017;12(7):e0180144.
32. Yee A, Webb T, Seaman A, Infantino M, Meacci F, Manfredi M, et al. Anti-CarP antibodies as promising marker to measure joint damage and disease activity in patients with rheumatoid arthritis. Immunol Res. 2015;61(1-2):24-30.
33. Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, McCormick JK, et al. Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. J Rheumatol. 2014;41(2):270-9.
34. Janssen KM, de Smit MJ, Brouwer E, de Kok FA, Kraan J, Altenburg J, et al. Rheumatoid arthritis-associated autoantibodies in non-rheumatoid arthritis patients with mucosal inflammation: a case-control study. Arthritis Res Ther. 2015;17:174.
35. Verheul MK, van Erp SJ, van der Woude D, Levarht EW, Mallat MJ, Verspaget HW, et al. Anti-carbamylated protein antibodies: a specific hallmark for rheumatoid arthritis. Comparison to conditions known for enhanced carbamylation; renal failure, smoking and chronic inflammation. Ann Rheum Dis. 2016;75(8):1575-6.

36. Montes A, Regueiro C, Perez-Pampin E, Boveda MD, Gomez-Reino JJ, Gonzalez A. Anti-Carbamylated Protein Antibodies as a Reproducible Independent Type of Rheumatoid Arthritis Autoantibodies. PLoS One. 2016;11(8):e0161141.
37. Chimenti MS, Triggianese P, Nuccetelli M, Terracciano C, Crisanti A, Guarino MD, et al. Auto-reactions, autoimmunity and psoriatic arthritis. Autoimmun Rev. 2015;14(12):1142-6.
38. Muller PC, Anink J, Shi J, Levarht EW, Reinards TH, Otten MH, et al. Anticarbamylated protein (anti-CarP) antibodies are present in sera of juvenile idiopathic arthritis (JIA) patients. Ann Rheum Dis. 2013;72(12):2053-5.
39. Bergum B, Koro C, Delaleu N, Solheim M, Hellvard A, Binder V, et al. Antibodies against carbamylated proteins are present in primary Sjogren's syndrome and are associated with disease severity. Ann Rheum Dis. 2016;75(8):1494-500.
40. Ziegelasch M, van Delft MA, Wallin P, Skogh T, Magro-Checa C, Steup-Beekman GM, et al. Antibodies against carbamylated proteins and cyclic citrullinated peptides in systemic lupus erythematosus: results from two well-defined European cohorts. Arthritis Res Ther. 2016;18(1):289.
41. Shi J, van Steenbergen HW, van Nies JA, Levarht EW, Huizinga TW, van der Helm-van Mil AH, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther. 2015;17:339.
42. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/. 2017.
43. Agresti A. On logit confidence intervals for the odds ratio with small samples. Biometrics. 1999;55(2):597-602.
44. Turunen S, Hannonen P, Koivula MK, Risteli L, Risteli J. Separate and overlapping specificities in rheumatoid arthritis antibodies binding to citrulline- and homocitrulline-containing peptides related to type I and II collagen telopeptides. Arthritis Res Ther. 2015;17:2.
45. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina AI, Israelsson L, et al. Antibodies to carbamylated alpha-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies. Arthritis Res Ther. 2016;18(1):96.
46. Li L, Deng C, Chen S, Zhang S, Wu Z, Hu C, et al. Meta-Analysis: Diagnostic Accuracy of Anti-Carbamylated Protein Antibody for Rheumatoid Arthritis. PLoS One. 2016;11(7):e0159000.
47. Skopelja S, Hamilton BJ, Jones JD, Yang ML, Mamula M, Ashare A, et al. The role for neutrophil extracellular traps in cystic fibrosis autoimmunity. JCI Insight. 2016;1(17):e88912.
48. Regueiro C, Nuno L, Ortiz AM, Peiteado D, Villalba A, Pascual-Salcedo D, et al. Value of Measuring Anti-Carbamylated Protein Antibodies for Classification on Early Arthritis Patients. Sci Rep. 2017;7(1):12023.
49. van Steenbergen HW, da Silva JAP, Huizinga TWJ, van der Helm-van Mil AHM. Preventing progression from arthralgia to arthritis: targeting the right patients. Nat Rev Rheumatol. 2018;14(1):32-41.
50. Mahler M. Population-based screening for ACPAs: a step in the pathway to the prevention of rheumatoid arthritis? Ann Rheum Dis. 2017;76(11):e42.
51. Deane KD, Striebich CC, Holers VM. Editorial: Prevention of Rheumatoid Arthritis: Now Is the Time, but How to Proceed? Arthritis Rheumatol. 2017;69(5):873-7.
52. Verheul MK, Shiozawa K, Levarht EW, Huizinga TW, Toes RE, Trouw LA, et al. Anti-carbamylated protein antibodies in rheumatoid arthritis patients of Asian descent. Rheumatology (Oxford). 2015;54(10):1930-2.