# Antibodies against advanced glycation end-products and malondialdehyde-acetaldehyde adducts identify a new specific subgroup of hitherto patients with seronegative arthritis with a distinct clinical phenotype and an HLA class II association

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ORIGINAL RESEARCH

# Antibodies against advanced glycation end-products and malondialdehyde-acetaldehyde adducts identify a new specific subgroup of hitherto patients with seronegative arthritis with a distinct clinical phenotype and an HLA class II association

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## ABSTRACT

**Objective** In rheumatoid arthritis (RA) around two-thirds of patients are autoantibody positive for rheumatoid factor, anti-citrullinated protein antibodies and/or anti-carbamylated protein antibodies. The remaining seronegative subgroup of patients is clinically heterogeneous and thus far, biomarkers predicting the disease course are lacking. Therefore, we analysed the value of other autoantibodies in RA directed against malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE).

**Methods** In sera of 648 patients with RA and 538 patients without RA from the Leiden Early Arthritis Clinic, anti-MAA and anti-AGE IgG antibody levels were measured using ELISA. Associations between genetic risk factors, acute phase reactants, radiological joint damage, remission and anti-PTM positivity were investigated using regression, correlation and survival analyses.

**Results** Anti-AGE and anti-MAA were most prevalent in RA (44.6% and 46.1% respectively) but were also present in non-RA arthritis patients (32.9% and 30.3% respectively). Anti-AGE and anti-MAA antibodies were associated with HLA-DRB1*03 within seronegative RA (OR=1.98, p=0.003, and OR=2.37, p<0.001, respectively) and, for anti-AGE also in non-RA arthritis patients (OR=2.34, p<0.001). Presence of anti-MAA antibodies was associated significantly with markers of inflammation, erythrocyte sedimentation rate and C reactive protein, in all groups independent of anti-AGE. Interestingly, the presence of anti-AGE and anti-MAA antibodies was associated with radiological progression in patients with seronegative RA, but not evidently with sustained drug-free remission.

**Conclusions** Anti-AGE and anti-MAA were present in around 45% of RA patients and 30% of non-RA arthritis patients, and although not specific for RA, their presence associated with HLA, inflammation and, for RA, with clinical outcomes especially in patients with seronegative RA.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Patients with rheumatoid arthritis (RA) can be divided into seropositive and seronegative subgroups. The presence of antibodies against post-translationally modified (PTM) proteins such as citrullinated proteins is nowadays used as a diagnostic and prognostic marker in RA. Antibodies directed against carbamylated proteins have more recently been shown to be present in a subset of the seronegative patients and are associated with bone erosions in that group.

## INTRODUCTION

In rheumatoid arthritis (RA) around two-thirds of patients are autoantibody positive for rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and/or anti-carbamylated protein (anti-CarP) antibodies.[1] The remaining seronegative subgroup of RA is clinically heterogeneous and thus far no reliable biomarkers are available to identify these patients or predict their disease course.[1]

ACPA and anti-CarP are antibodies that recognise proteins that have undergone post-translational modification (PTM), citrullination of arginine and carbamylation of lysine respectively.[2 3] However, many other types

### WHAT THIS STUDY ADDS

⇒ In this study, two different anti-PTM antibodies are investigated: anti-advanced glycation end-product modified protein antibodies (anti-AGE) and anti-malondialdehyde-acetaldehyde adduct modified protein antibodies (anti-MAA). These antibodies can be detected in several forms of inflammatory arthritis. Within seronegative RA (negative for rheumatoid factor, anti-citrullinated protein antibodies and anti-carbamylated protein antibodies), 16.9% of patients are positive for anti-MAA and/or anti-AGE antibodies. This subgroup is characterised by an association with HLA-DRB1*03, increased radiographic joint damage and (for anti-MAA) inflammation.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The presence of anti-PTM antibodies like anti-AGE and anti-MAA in patients with RA and other patients with inflammatory arthritis previously considered to be seronegative, may not only serve as a prognostic marker, but importantly may contribute to understanding the pathogenesis of these conditions, including a subset of RA.

of PTMs exist.[4] Two examples of PTMs that are found to associate with disease are advanced glycation end-products (AGE) and malondialdehyde-acetaldehyde adducts (MAA). AGEs are a result of oxidative stress and tissue damage[5] and are, for example, present in patients with diabetes mellitus type 2.[6] Interestingly, in these patients also antibodies directed against this PTM were observed.[6] MAA modifications are a result of reactive oxygen species that are formed during inflammation and oxidative stress.[7] Both MAA-modified proteins and anti-MAA antibodies are found in patients with RA, as well as in other diseases.[7] AGE and MAA are both highly immunogenic PTMs.[8 9] Therefore, it is plausible that antibodies against AGE and MAA are also present in patients with arthritis.

Seronegative RA is associated with HLA-DRB1*03, suggesting a role for immunopathology driven by, for example, B cell immunity.[10] Indeed, within the ACPA-negative patients, the presence of anti-CarP was associated with HLA-DRB1*03.[11 12] However, it did not yet explain the full HLA-DRB1*03 association, raising the possibility that other anti-PTM responses may be present in 'seronegative' RA that are present in the remainder of the HLA-DRB1*03 positive individuals.[13 14] On top of this haplotype association, within these patients with ACPA-negative RA, anti-CarP was found to associate with a more severe radiological progression.[3] Patients with seronegative RA are a diverse group of patients that in many ways resemble undifferentiated arthritis. Presence of antibodies, like anti-PTM antibodies, might help to better understand and characterise subgroups that possibly belong to this so-called seronegative RA.

We therefore investigated whether anti-AGE and anti-MAA antibodies are present in patients with RA and other forms of arthritis, and whether they could potentially close the so-called serological gap[1] in seronegative RA.

## METHODS

### Patients

One thousand one hundred eighty-six patients with arthritis of at least one joint and a symptom duration of less than 2 years were included in the Leiden Early Arthritis Clinic (EAC) cohort.[15] Data were collected at baseline and follow-up (4, 12 months and yearly thereafter). Patients were being followed as long as the patient remained being seen clinically by the rheumatologist. RA was classified based on the 1987 American College of Rheumatology criteria (n=648).[16] Definitive diagnoses other than RA (n=538) were made by the treating physician after 1 year of follow-up and were predominantly psoriatic arthritis (PsA) (n=100), inflammatory osteoarthritis (n=95) and gout (n=93) besides other more rare forms of arthritis. For this manuscript, the following diagnoses were termed autoimmune (AI): RA, PsA, spondyloarthritis, sarcoidosis, systemic lupus erythematosus (SLE) and paraneoplastic arthritis. The diagnoses termed as non-autoimmune (non-AI) were: gout, pseudogout and septic arthritis. Clinical and demographic patient characteristics were collected as described previously.[17]

### Genotyping, radiological progression and remission

From all patients, HLA genotypes were established as described previously.[18] The alleles that were marked as shared epitope-encoding HLA (HLA-SE) positive were: HLA-DRB1*01:01, 01:02, 04:01, 04:04, 04:05, 04:08, 10:01 and 14:02. For the radiological progression analyses, 2853 X-ray sets of the hands and feet of 635 patients with RA were scored as described previously using the Sharp-van der Heijde score (SHS).[19 20] Sustained drug-free remission (SDFR) was defined as the absence of clinical synovitis after discontinuation of disease-modifying antirheumatic drug treatment, that persisted for the entire follow-up, being at least 1 year.[21]

### Anti-AGE and anti-MAA measurements

Anti-AGE and anti-MAA antibodies were detected using an in-house ELISA based on modified fetal calf serum (FCS) as described previously.[22] Briefly, modified and non-modified FCS were coated to a Nunc Maxisorp ELISA plate (430341, Thermofisher). In between each sequential step, plates were washed three times using phosphate buffered saline (PBS)/0.05%Tween (Sigma, P1379). After blocking (PBS/1%bovine serum albumin) for 6 hours at 4°C plates were incubated overnight at 4°C with 1/100 or 1/1000 diluted serum for anti-AGE and anti-MAA, respectively. Each plate contained a standard of anti-PTM positive serum to calculate arbitrary units. After incubation, IgG levels were detected using Rabbit-anti-Human IgG-HRP (Dako, P0214). Plates were developed by incubating with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)/0.015% $H_2O_2$ (A1888 and 7722-84-1, both from Merck) and absorbance at 415 nm was

Table 1 Baseline characteristics of the rheumatoid arthritis (RA), non-RA, autoimmune no RA and non-autoimmune group

| | RA (n=648)* | non-RA (n=538)* | AI no RA (n=233)* | non-AI (n=226)* |
|---|---|---|---|---|
| Female (n,%) | 432 (66.7%) | 269 (50.0%) | 165 (49.8%) | 112 (49.6%) |
| Age (mean, SD) | 57.3 (17.4) | 50.9 (15.8) | 43.9 (15.7) | 61.2 (13.5) |
| BMI (mean, SD) | 25.9 (3.9) | 26.5 (4.5) | 25.7 (4.3) | 27.5 (4.4) |
| Sympt. dur. weeks (median, IQR) | 18 (9–36) | 9 (2–27) | 11 (4–28) | 10 (2–31) |
| SJC (in 28 joints) (median, IQR) | 6 (3–11) | 1 (1–4) | 2 (0–4) | 1 (1–4) |
| TJC (in 28 joints) (median, IQR) | 8 (4–14) | 4 (1–9) | 5 (2–9) | 4 (1–8) |
| VAS (0–100) (median, IQR) | 42 (20–58) | 40 (19–60) | 40 (20–60) | 35 (19–52) |
| ESR (median, IQR) | 34 (19–54) | 27 (11–50) | 33 (13–56) | 19 (9–37) |
| CRP (median, IQR) | 18 (8–41) | 13 (4–34) | 18 (6–41) | 9 (3 – 23) |
| HAQ (median, IQR) | 1 (0.62–1.62) | 0.75 (0.25–1.13) | 0.63 (0.25–1.13) | 0.75 (0.25–1.13) |
| Smoking+ (n,%) | 159 (24.5%) | 102 (19.0%) | 63 (20.5%) | 41 (20.8%) |
| HLA-SE+ (n,%) | 410 (63.3%) | 128 (23.8%) | 411 (48.5%) | N/A† |
| ACPA | 317 (51.3%) | 22 (5.1%) | 17 (7.0%) | 5 (2.7%) |
| RF | 365 (56.3%) | 52 (9.8%) | 28 (10.4%) | 24 (10.9%) |

Diagnoses were termed non-RA: all diagnoses other than RA within the EAC cohort. Diagnoses were termed autoimmune (AI) no RA: psoriatic arthritis, spondyloarthritis, sarcoidosis, SLE and paraneoplastic arthritis. Diagnoses termed as non-autoimmune (non-AI) were: gout, pseudogout and septic arthritis.
*Numbers differ slightly per analyses due to missing variables.
†Data not shown (47.8% missing).
BMI, body mass index; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; HLA-SE, HLA Shared Epitope; SJC, swollen joint count; Sympt. Dur. Weeks, symptom duration in weeks; TJC, tender joint count; VAS, visual analog scale.

measured using a microplate reader (Bio-Rad iMark). The cut-off for positivity was set as the mean arbitrary units plus two times the SD of 80 healthy controls, excluding values higher than 10× the mean.

### Statistical analysis

Independent samples t-test and Mann-Whitney U tests were used to analyse the baseline characteristics. The association of HLA-DRB1*03 with autoantibodies was assessed with logistic regression, and stratified for anti-cyclic citrullinated peptide 2 (anti-CCP2) and anti-CarP if relevant. Correlations between anti-PTM antibodies and inflammatory markers were calculated using Spearman's rank correlation. For the radiological progression analyses, a multivariate normal regression model for longitudinal data was used with SHS as response variable. The model controlled for the age, sex and inclusion year of the patients.[19] SDFR development until follow-up was calculated using Kaplan-Meier survival analysis and Cox's regression. All statistical analysis were performed using SPSS statistics V.25 (IBM).

## RESULTS

### Anti-AGE and anti-MAA in patients with arthritis

Baseline characteristics are described in table 1. Anti-PTM antibody levels were measured in RA and non-RA arthritis patients and compared with healthy controls (figure 1A,B and online supplemental table 1). The non-RA arthritis group was divided into subgroups and separately depicted based as AI arthritis (without RA) including PsA, paraneoplastic arthritis, SLE, sarcoidosis and spondyloarthritis and as non-AI arthritis including septic arthritis, gout and pseudogout.

Compared with healthy controls, anti-AGE and anti-MAA were most prevalent in RA (anti-AGE: 7.5% in HC vs 44.6% in RA and anti-MAA: 3.8% in HC vs 46.1% in RA) but were also present in other types of early arthritis. Within patients without RA, anti-AGE and anti-MAA were present in 32.9% and 30.3%, respectively and in non-RA AI arthritis anti-AGE and anti-MAA were found in 38.5% and 41.5%, respectively. These data indicate that the presence of anti-PTM antibodies is not specific for RA. When analysing combinations of autoantibodies, the largest subgroup of patients with RA (n=99) had all four anti-PTM antibodies (anti-AGE, anti-MAA, anti-CarP, anti-CCP2) as well as RF, after which the second largest group (n=63) was characterised by the combination of RF, anti-CCP2 and anti-CarP (figure 1C).

Interestingly, 67 (34.0%) and 57 (28.9%) of patients with seronegative (RF negative, ACPA negative and anti-CarP negative) RA were positive for anti-AGE and anti-MAA, respectively. Moreover, 40 (20.3%) of these patients with seronegative RA were positive for both anti-AGE and anti-MAA. These anti-PTM responses may identify a new subgroup in the patients with otherwise seronegative RA.

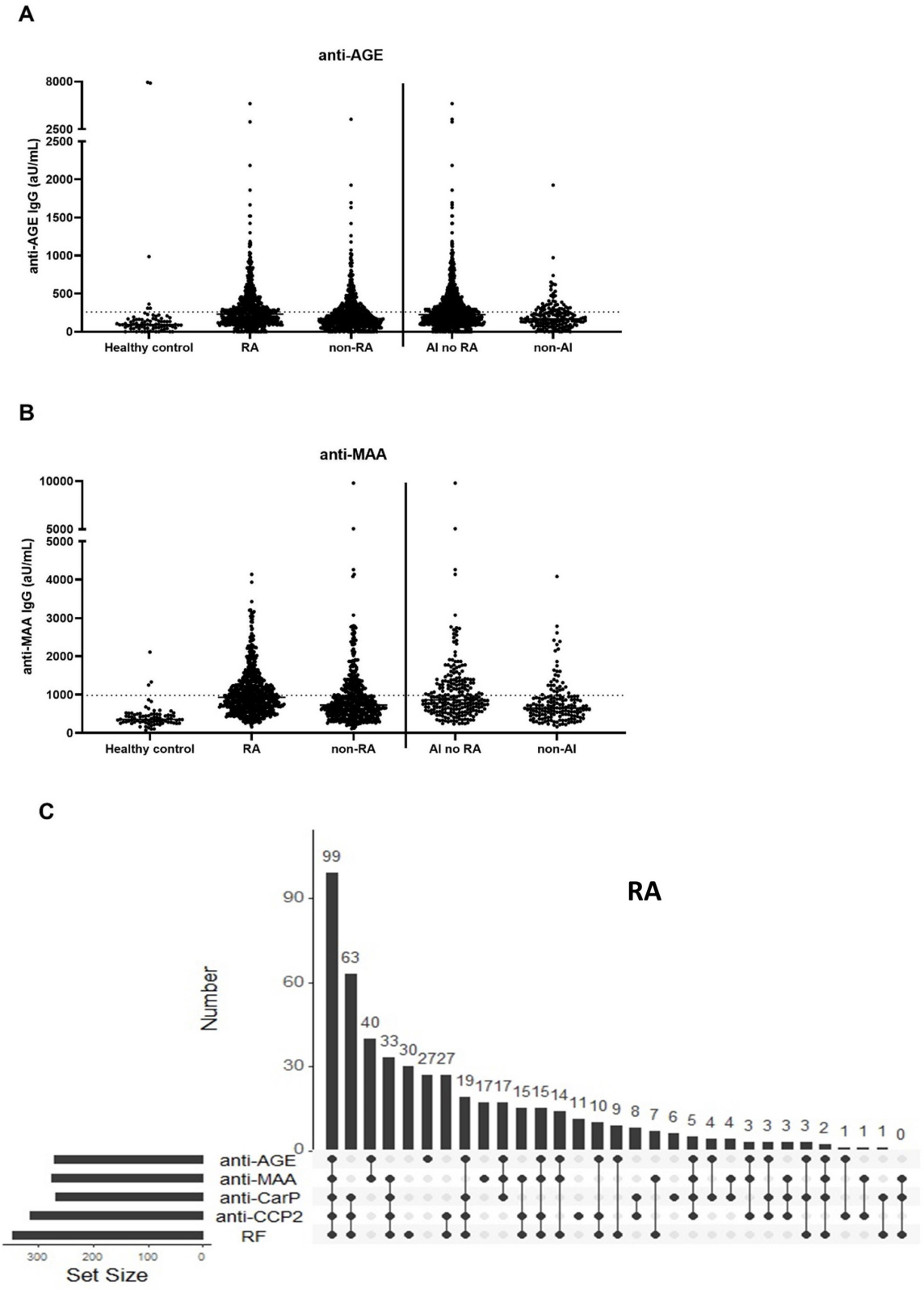



**Figure 1** Anti-AGE and anti-MAA show higher levels in RA and occur in a subgroup of patients with anti-CarP anti-CCP2 negative RA. IgG antibody levels of anti-AGE (A) and anti-MAA (B) in patients with (n=648) and without (n=538) RA. Early patients with arthritis were separately depicted as groups: AI without RA (including psoriatic arthritis, paraneoplastic arthritis, SLE, sarcoidosis and spondyloarthritis) and non-AI (including septic arthritis, gout, pseudogout). (C) Upset plots of groups of patients with RA (n=499*) positive for anti-PTM combinations; anti-AGE, anti-MAA, anti-CarP, anti-CCP2 and RF. *Data for anti-CarP was missing for 149 patients with RA. AGE, advanced glycation end-product; AI, autoimmune; aU/mL, arbitrary units per mL; CarP, carbamylated protein; CCP2, citrullinated cyclic peptide 2; MAA, malondialdehyde acetaldehyde adduct; RA, rheumatoid arthritis; RF, rheumatoid factor.

### HLA-DRB1*03 associates with anti-AGE and anti-MAA independently of anti-CarP in patients with anti-CCP2-negative RA

Since HLA class II alleles are known to associate with autoantibody positivity in RA, we sought to investigate the presence of HLA-SE and its association with anti-AGE and anti-MAA antibodies. Of all patients with RA, 63.3% were HLA-SE+ (table 1). Based on the well-known association between HLA-SE and RA, the HLA-SE alleles were assessed and were significantly more prevalent in all RA subgroups compared with healthy controls. In the anti-AGE-positive group, as compared with patients with anti-AGE-negative RA however, the prevalence of HLA-SE alleles was similar (table 2). The same was true for anti-MAA; therefore, both anti-AGE and anti-MAA antibodies were not associated with HLA-SE.

Since HLA-DRB1*03 is associated with seronegative RA and anti-CarP antibodies in this disease subset, we sought to investigate the association of HLA-DRB1*03 with anti-AGE and anti-MAA. In patients with RA, HLA-DRB1*03 was more prevalent in anti-AGE-positive and anti-MAA-positive patients as compared with healthy controls with OR values of 1.34 (95% CI 1.01 to 1.78, p=0.05) and 1.29 (95% CI 0.96 to 1.73, p=0.09), although this did not achieve statistical significance compared with anti-AGE-negative or anti-MAA-negative patients, respectively (table 2, part I). To investigate whether HLA-DRB1*03 is associated with anti-MAA and anti-AGE in anti-CCP2-negative RA, we focused on this subset and stratified the analysis for anti-CarP. Within the patients with anti-CCP2 negative RA, anti-AGE and anti-MAA antibodies were associated with HLA-DRB1*03 compared with healthy controls (OR: 1.98, 95% CI 1.27 to 3.07, p=0.003, and OR: 2.37, 95% CI 1.50 to 3.74, p<0.001, respectively). Anti-MAA was associated with HLA-DRB1*03 in the anti-CCP2 negative stratum independent of anti-CarP (OR: 1.91, 95% CI 1.11 to 3.30, p=0.02) (table 2, part II). In this stratified analysis, anti-AGE showed the same trend for association but did not reach significance (OR: 1.48, 95% CI 0.86 to 2.52, p=0.16). Since anti-AGE and anti-MAA often co-occur, we next stratified the association analysis for these autoantibodies, to dissect whether the observed association to HLA-DRB1*03 could be attributed to one of them in particular. After stratification for anti-AGE or anti-MAA, only patients with double positive RA showed a significant association with HLA-DRB1*03 compared with healthy controls (online supplemental table 2, part I). Since some controversy exists on the association of HLA-DRB1*03 in patients with anti-CCP2 negative RA, we investigated the association between anti-AGE and anti-MAA with HLA-DRB1*03 within patients with HLA-SE negative RA. In both HLA-SE negative and anti-CCP2 negative stratum, we find similar associations with anti-AGE/-MAA and HLA-DR1*03 (table 2, part III).

In non-RA arthritis patients, both anti-AGE and anti-MAA showed a similar association with HLA-DRB1*03 with OR values of 2.34 (95% CI 1.58 to 3.47, p<0.001) and 1.94 (95% CI 1.29 to 2.92, p=0.002) compared with healthy controls (table 2, part I). In a comparison within the non-RA arthritis patients, HLA DRB1*03 remained significantly associated with anti-AGE-positive compared with anti-AGE-negative patients (OR: 2.22, 95% CI 1.28 to 3.84, p=0.01), while the association with anti-MAA did not remain significant. To disentangle the effects of anti-AGE and anti-MAA, analyses were again stratified, after which only the presence of anti-AGE in patients with anti-MAA-negative without RA remained significantly associated with HLA-DRB1*03 (online supplemental table 2, part II).

Taken together, these data indicate that anti-AGE and anti-MAA associate with HLA-DRB1*03 in RA and non-RA arthritis patients, and that this association (which cannot be ascribed to anti-AGE or anti-MAA in particular) is mainly present in patients with anti-CCP2 negative RA. Similar associations were observed in patients with HLA-SE negative RA.

### Inflammation markers associate with anti-MAA positivity in RA and non-RA arthritis

Next, we sought to investigate whether anti-PTM antibodies correlate with inflammation markers erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) (table 3). Higher inflammation parameters in anti-AGE- and anti-MAA-positive individuals were observed in RA and non-RA arthritis patients, and in both the autoimmune and non-autoimmune subgroups of patients with arthritis. To investigate whether both anti-MAA and anti-AGE were associated with acute phase reactants in RA independently, anti-AGE and anti-MAA were stratified for each other. After this stratification, anti-AGE was no longer associated with either CRP or ESR whereas the association of anti-MAA with these inflammation markers remained significant (online supplemental table 3). These data indicate that anti-PTM responses, especially anti-MAA, is associated with markers of inflammation in early arthritis in both RA and non-RA arthritis patients.

### Anti-AGE and anti-MAA associate with radiological progression in patients with anti-CCP2-negative RA

We next analysed if the presence of anti-AGE and anti-MAA is associated with radiological progression in RA. Anti-AGE-positive patients displayed more radiographic damage per year than anti-AGE-negative patients (p<0.001) (figure 2A). Data were then stratified for anti-CCP2, which revealed that this association was mainly present in the anti-CCP2-negative subgroup (figure 2B). When anti-CCP2 negative patients were further stratified for anti-CarP, the association between anti-AGE and radiographic progression remained significant (figure 2C). This indicates that in patients with anti-CCP2 negative RA, anti-AGE is associated with radiological progression independent of anti-CarP, suggesting that this anti-PTM antibody could discriminate a different subgroup. Anti-MAA positivity was also associated with radiological progression (p=0.002) (figure 2D). This effect was also observed

**Table 2** Association between anti-AGE and anti-MAA antibodies and HLA-SE and HLA-DRB1*03 presence in RA and HLA-DRB1*03 presence in non-RA patients from the Leiden EAC cohort

| | RA n=648 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Part I** | **HLA-SE–** | **HLA-SE+** | **Total** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Healthy controls | 674 (55.7) | 537 (44.3) | 1211 | 1 (ref) | | | | | |
| Anti-AGE– | 139 (38.7%) | 220 (61.3%) | 359 | **1.99** | (**1.56 to 2.53**) | **<0.001** | 1 (ref) | | |
| Anti-AGE+ | 99 (34.3%) | 190 (65.7%) | 289 | **2.41** | (**1.84 to 3.15**) | **<0.001** | 1.21 | (0.88 to 1.67) | 0.24 |
| Anti-MAA– | 130 (37.2%) | 219 (62.8%) | 349 | **2.11** | (**1.66 to 2.70**) | **<0.001** | 1 (ref) | | |
| Anti-MAA+ | 108 (36.1%) | 191 (63.9%) | 299 | **2.22** | (**1.71 to 2.88**) | **<0.001** | 1.04 | (0.76 to 1.45) | 0.77 |
| | **HLA-DRB1*03–** | **HLA-DRB1*03+** | **Total** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Healthy controls | 941 (77.7%) | 270 (22.3%) | 1211 | 1 (ref) | – | – | – | – | – |
| Anti-AGE– | 271 (75.5%) | 88 (24.5%) | 359 | 1.13 | (0.86 to 1.49) | 0.38 | 1 (Ref) | – | – |
| Anti-AGE+ | 211 (73.0%) | 78 (27.0%) | 289 | 1.29 | (0.96 to 1.73) | 0.09 | 1.14 | (0.80 to 1.62) | 0.47 |
| Anti-MAA– | 266 (76.2%) | 83 (23.8%) | 349 | 1.09 | (0.82 to 1.44) | 0.56 | 1 (ref) | – | – |
| Anti-MAA+ | 216 (72.2%) | 83 (27.8%) | 299 | **1.34** | (**1.01 to 1.78**) | **0.05** | 1.23 | (0.87 to 1.75) | 0.25 |
| | **non-RA n=246*** | | | | | | | | |
| | **HLA-DRB1*03–** | **HLA-DRB1*03+** | **Total** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Healthy controls | 941 (77.7%) | 270 (22.3%) | 1211 | 1 (ref) | – | – | – | – | – |
| Anti-AGE– | 99 (76.7%) | 30 (23.3%) | 129 | 1.06 | (0.69 to 1.62) | 0.80 | 1 (Ref) | – | – |
| Anti-AGE+ | 70 (59.8%) | 47 (40.2%) | 117 | **2.34** | (**1.58 to 3.47**) | **<0.001** | **2.22** | (**1.28 to 3.84**) | **0.01** |
| Anti-MAA– | 97 (72.4%) | 37 (27.6%) | 134 | 1.33 | (0.89 to 1.99) | 0.17 | 1 (ref) | – | – |
| Anti-MAA+ | 72 (64.3%) | 40 (35.7%) | 112 | **1.94** | (**1.29 to 2.92**) | **0.002** | 1.46 | (0.85 to 2.50) | 0.17 |
| | **anti-CCP2-negative RA n=301†** | | | | | | | | |
| **Part II** | **HLA-DRB1*03–** | **HLA-DRB1*03+** | **Total** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Healthy controls | 941 (77.7%) | 270 (22.3%) | 1211 | 1 (ref) | – | | | | |
| Anti-AGE–Anti-CarP– | 125 (72.3%) | 48 (27.7%) | 173 | 1.34 | (0.93 to 1.92) | 0.11 | 1 (ref) | – | – |
| Anti-AGE+Anti-CarP– | 60 (63.8%) | 34 (36.2%) | 94 | **1.98** | (**1.27 to 3.07**) | **0.003** | 1.48 | (0.86 to 2.52) | 0.16 |
| Anti-AGE–Anti-CarP+ | 6 (50.0%) | 6 (50.0%) | 12 | **3.49** | (**1.11 to 10.89**) | **0.03** | 2.60 | (0.80 to 8.47) | 0.11 |
| Anti-AGE+Anti-CarP+ | 14 (50.0%) | 14 (50.0%) | 28 | **3.49** | (**1.64 to 7.40**) | **0.001** | **2.60** | (**1.16 to 5.87**) | **0.02** |
| | **anti-CCP2-negative RA n=307‡** | | | | | | | | |
| | **HLA-DRB1*03–** | **HLA-DRB1*03+** | **Total** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Healthy controls | 941 (77.7%) | 270 (22.3%) | 1211 | 1 (ref) | – | | | | |
| Anti-MAA–Anti-CarP– | 135 (73.8%) | 48 (26.2%) | 183 | 1.24 | (0.87 to 1.77) | 0.24 | 1 (ref) | – | – |
| Anti-MAA+Anti-CarP– | 50 (59.5%) | 34 (40.5%) | 84 | **2.37** | (**1.50 to 3.74**) | **<0.001** | **1.91** | (**1.11 to 3.30**) | **0.02** |
| Anti-MAA–Anti-CarP+ | 6 (42.9%) | 8 (57.1%) | 14 | **4.65** | (**1.60 to 13.51**) | **0.01** | **3.75** | (**1.24 to 11.36**) | **0.02** |
| Anti-MAA+Anti-CarP+ | 14 (53.8%) | 12 (46.2%) | 26 | **2.99** | (**1.37 to 6.54**) | **0.01** | **2.41** | (**1.04 to 5.58**) | **0.04** |

Continued

**Table 2** Continued

| Part III | SE-negative RA n=271† § | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **HLA-DRB1*03–** | **HLA-DRB1*03+** | **Total** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Healthy controls | 941 (77.7%) | 270 (22.3%) | 1211 | 1 (ref) | – | – | | | |
| Anti-AGE–Anti-CarP– | 110 (82.7) | 23 (17.3) | 133 | 0.73 | 0.46 to 1.17) | 0.29 | 1 (ref) | – | – |
| Anti-AGE+Anti-CarP– | 36 (58.1) | 26 (41.9) | 62 | **2.52** | **(1.49 to 4.24)** | **<0.001** | **3.45** | **(1.76 to 6.79)** | **<0.001** |
| Anti-AGE–Anti-CarP+ | 23 (59.0) | 16 (41.0) | 39 | **2.42** | **(1.26 to 4.66)** | **0.008** | **3.33** | **(1.52 to 7.26)** | **0.003** |
| Anti-AGE+Anti-CarP+ | 23 (62.2) | 14 (37.8) | 37 | **2.12** | **(1.08 to 4.18)** | **0.03** | **2.91** | **(1.31 to 6.49)** | **0.009** |
| | **SE-negative RA n=269¶** | | | | | | | | |
| | **HLA-DRB1*03–** | **HLA-DRB1*03+** | **Total** | **OR** | **95% CI** | **P value** | **OR** | **95% CI** | **P value** |
| Healthy controls | 941 (77.7%) | 270 (22.3%) | 1211 | 1 (ref) | – | – | | | |
| Anti-MAA–Anti-CarP– | 114 (85.7%) | 19 (14.3) | 133 | **0.58** | **(0.35 to 0.96)** | **0.04** | 1 (ref) | – | – |
| Anti-MAA+Anti-CarP– | 35 (58.3%) | 25 (41.7) | 60 | **2.49** | **(1.46 to 4.23)** | **<0.001** | **4.29** | **(2.11 to 8.69)** | **<0.001** |
| Anti-MAA–Anti-CarP+ | 15 (51.7) | 14 (48.3) | 29 | **3.25** | **(1.56 to 6.82)** | **0.002** | **5.60** | **(2.33 to 13.44)** | **<0.001** |
| Anti-MAA+Anti-CarP+ | 31 (66.0) | 16 (34.0) | 47 | 1.80 | (0.97 to 3.34) | 0.06 | **3.10** | **(1.43 to 6.72)** | **0.004** |

Statistically significant difference between patient group and healthy controls (p≤0.05).
*HLA-DRB1*03 data were present from 246 of 538 patients without RA.
†HLA-DRB1*03 as well as anti-CarP and anti-AGE data was present from 301 of 648 patients with RA.
‡HLA-DRB1*03 as well as anti-CarP and anti-MAA data was present from 307 of 648 patients with RA.
§HLA-SE as well as anti-CarP and anti-AGE data was present from 271 of 648 patients with RA.
¶HLA-SE as well as anti-CarP and anti-MAA data was present from 269 of 648 patients with RA.
AGE, advanced glycation end-products; CarP, carbamylated protein; CCP2, citrullinated cyclic peptide 2; HLA-SE, human leucocyte antigen shared epitope; MAA, malondialdehyde acetaldehyde adducts; RA, rheumatoid arthritis.

**Table 3** Association between anti-AGE and anti-MAA antibin RA and non-RA arthritis patientsodies and ESR and CRP levels

| | RA n=648* | | non-RA n=538* | | AI no RA n=233* | | non-AI n=131* | |
|---|---|---|---|---|---|---|---|---|
| | ESR (median, IQR) | CRP (median, IQR) | ESR (median, IQR) | CRP (median, IQR) | ESR (median, IQR) | CRP (median, IQR) | ESR (median, IQR) | CRP (median, IQR) |
| n, % anti-AGE+ | 289 (44.6%) | | 177 (32.9%) | | 90 (38.6%) | | 31 (23.7%) | |
| Anti-AGE− | 32.0 (19.0–52.8) | 17.0 (7.0–36.0) | 21.5 (9.0–41.0) | 11.0 (3.8–29.0) | 24.0 (10.0–47.0) | 14.0 (4.0–32.0) | 22.0 (9.0–93.0) | 14.0 (4.0–34.0) |
| Anti-AGE+ | 38.0 (19.0–57.0) | 19.0 (9.0–48.0) | 39.0 (21.5–59.5) | 19.0 (7.0–55.0) | 43.5 (28.5–64.8) | 21.0 (9.0–56.0) | 42.0 (26.0–55.0) | 25.0 (6.5–93.0) |
| P value | **0.02** | **0.03** | **<0.001** | **<0.001** | **<0.001** | **0.004** | **0.001** | 0.095 |
| n, % anti-MAA+ | 299 (46.1%) | | 163 (30.3%) | | 96 (41.2%) | | 19 (14.5%) | |
| Anti-MAA− | 30.0 (16.8–48.3) | 15.0 (6.0–31.0) | 20.5 (9.0–39.0) | 10.0 (3.0–27.0) | 25.0 (10.0–45.0) | 13.0 (4.0–32.0) | 22.0 (11.0–41.0) | 13.8 (4.0–34.0) |
| Anti-MAA+ | 41.0 (22.0–61.5) | 22.5 (10.0–48.3) | 42.0 (27.0–61.0) | 21.0 (9.0–48.8) | 44.5 (27.0–62.5) | 21.5 (8.8–56.0) | 45.0 (31.0–58.0) | 25.0 (9.0–79.2) |
| P value | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.003** | **0.005** | 0.06 |

Statistically significant difference between groups (p≤0.05).
*ESR and CRP levels were not determined for all patients and numbers might therefore slightly differ per variable.
AGE, advanced glycation end-products; AI, autoimmune (RA, psoriatic arthritis, paramalignant arthritis, SLE, sarcoidosis, spondyloarthropathy); CRP, C reactive protein; ESR, erythrocyte sedimentation rate; MAA, malondialdehyde acetaldehyde adducts; non-AI, non-autoimmune (septic arthritis, gout and pseudogout); RA, rheumatoid arthritis.

in the anti-CCP2-negative stratum (figure 2E), although no longer significant after stratifying for anti-CCP2. The latter could be a consequence of power as the effect size (beta) which decreased only slightly to 1.03/year, p=0.16 (figure 2E).

### Presence of anti-MAA or anti-AGE is not associated with SDFR in RA

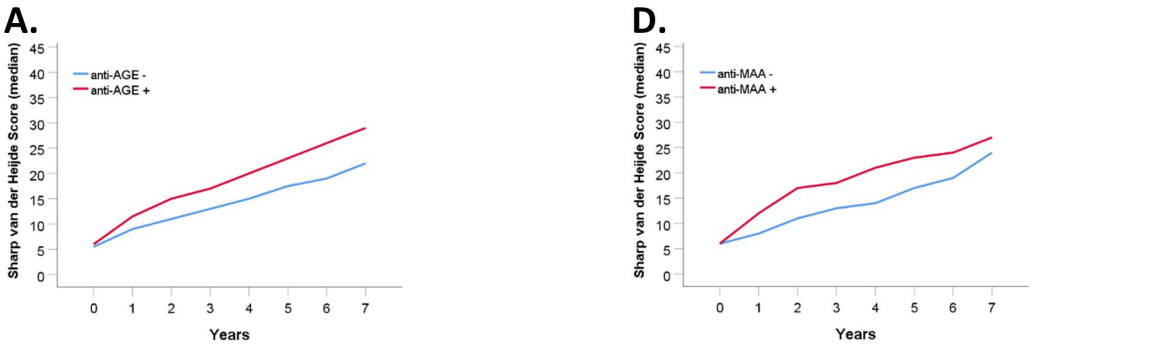
Next, we sought to investigate whether anti-AGE and anti-MAA were associated with SDFR over time (online supplemental figure 3). Anti-AGE was not associated with SDFR, HR 0.93 (95% CI 0.66 to 1.30; p=0.66) which did not differ after adjusting for CCP2 status (HR 1.14, 95% CI 0.81 to 1.61, p=0.46). Anti-MAA-positive patients were less likely to achieve SDFR, compared with anti-MAA-negative patients, HR 0.72 (95% CI 0.51 to 1.00, p=0.053). After adjusting for CCP2 status, there was no longer an association between anti-MAA and SDFR, HR 1.05 (95% CI 0.74 to 1.50, p=0.80).

## DISCUSSION

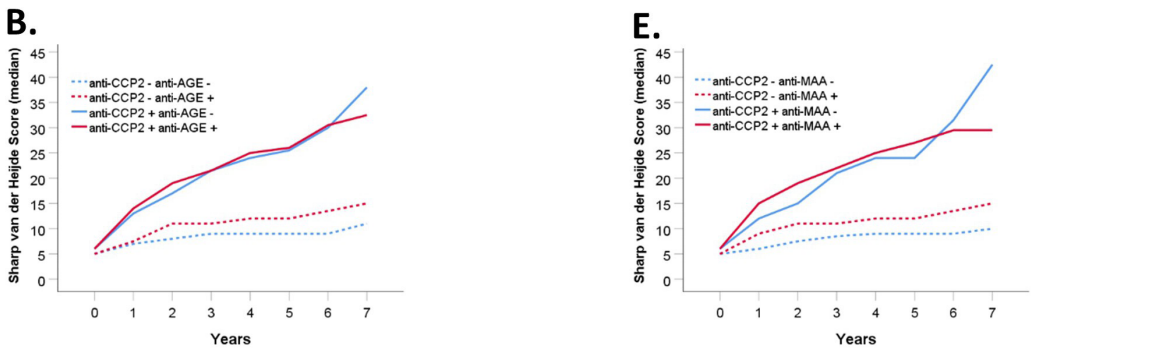
In this study, we demonstrated that anti-AGE and anti-MAA are present in patients with RA, and interestingly also in a substantial part of patients with otherwise seronegative RA. This is not specific for RA, as anti-AGE and anti-MAA antibodies were also present in other forms of early arthritis. Both anti-AGE and anti-MAA are associated with HLA-DRB1*03 in RA, and anti-AGE is also associated with HLA-DR1*03

in non-RA arthritis patients. Anti-AGE and anti-MAA are associated with a distinct clinical phenotype: anti-AGE associates with radiological progression in RA whereas anti-MAA only showed a trend with radiological progression but associated with increased inflammatory parameters in both RA and non-RA arthritis.

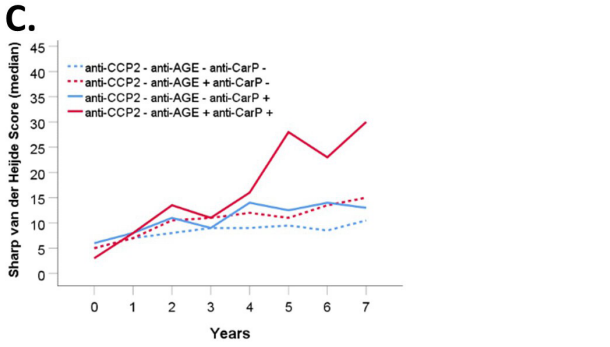
Associations with particular HLA class II alleles have been described to occur in many seropositive AI diseases.[13 14] More specifically, HLA-DRB1*03, initially reported to be associated with anti-CCP2 negative RA, was later associated with the presence of anti-CarP, although not all HLA-DRB1*03-positive patients were anti-CarP-positive.[10 12] In this study, we observed that HLA-DRB1*03 was associated with anti-AGE and anti-MAA in patients with anti-CCP2 negative RA which was independent of anti-CarP, thereby identifying another subgroup of anti-CCP2 negative RA that is associated with HLA-DRB1*03. In addition, anti-AGE associated with HLA-DRB1*03 in non-RA confirming the robustness of this finding. Together, these observations provide additional insight into the association of HLA-DBR1*03 with (rheumatoid) arthritis; although these alleles are not associated with the presence of ACPA, they do appear to predispose to the formation of other autoantibodies (anti-CarP, anti-AGE and anti-MAA) in a process in which HLA class II-associated T-cell-dependent immune responses are likely to be involved.




| | | | |
|---|---|---|---|
| Anti-AGE+ | 1.05 (1.03, 1.07), p<0.001 | Anti-MAA+ | 1.04 (1.01, 1.06), p=0.002 |




| | | | | | |
|---|---|---|---|---|---|
| CCP2- Anti-AGE- | Reference | | CCP2- Anti-MAA- | Reference | |
| CCP2+ Anti-AGE- | 1.12 (1.09, 1.16), p<0.001 | Reference | CCP2+ Anti-MAA- | 1.12 (1.09, 1.16), p<0.001 | Reference |
| CCP2- Anti-AGE+ | 1.04 (1.003, 1.08), p=0.03 | | CCP2- Anti-MAA+ | 1.03 (0.99, 1.06), p=0.16 | |
| CCP2+ Anti-AGE+ | 1.14 (1.11, 1.18), p<0.001 | 1.02 (0.99, 1.05), p=0.26 | CCP2+ Anti-MAA+ | 1.13 (1.10, 1.16), p<0.001 | 1.01 (0.97, 1.04), p=0.68 |




| | | |
|---|---|---|
| CCP2- Anti-AGE-Anti-CarP- | Reference | |
| CCP2- Anti-AGE+Anti-CarP- | 1.04 (1.003, 1.95), p-0.027 | |
| CCP2- Anti-AGE-Anti-CarP+ | 1.04 (0.97, 1.12), p=0.26 | Reference |
| CCP2- Anti-AGE+Anti-CarP+ | 1.12 (1.06, 1.18), p<0.001 | 1.04 (10004, 1.92), p=0.03 |

**Figure 2** Anti-AGE and anti-MAA associate with radiological progression in patients with RA (n=600). (A) Radiological progression in anti-AGE positive and negative RA. (B) Data stratified for CCP2. (C) Data stratified for anti-CarP in anti-CCP2-negative stratum. (D) Radiological progression in anti-MAA positive and negative RA. (E) Data stratified for CCP2. Data presented as estimate (95% CI), p value. AGE, advanced glycation end-product; CarP, carbamylated protein; CCP2, citrullinated cyclic peptide 2; MAA, malondialdehyde acetaldehyde adduct.

Interestingly, in RA, anti-AGE associated with radiological progression independent of anti-CCP2 and anti-CarP suggesting an additive value of anti-AGE in determining disease evolution as it could define a new subgroup of patients with RA. Strikingly, anti-AGE was not associated with SDFR. In RA and non-RA, a subgroup of patients is characterised by more extensive inflammation and the presence of anti-MAA antibodies, while a subgroup of patients with CCP2-negative RA is characterised by radiological progression and presence of anti-AGE antibodies. Based on these results, distinct subgroups within RA and non-RA can be delineated based on their specific clinical phenotype.

The presence of AGE-modified proteins and anti-AGE antibodies has been observed in diabetes and hypertension.[6 23] Also, in synovial tissue and sera of patients with RA, AGE-modified proteins have been detected.[24–26] In addition, MAA-modified proteins have been observed before in RA tissue[7] and it is clear that both modifications can be induced by inflammation and oxidative

stress in the inflamed joint.[5 7] Our study now adds that in a subset of the patients with RA antibodies against these PTMs are present. Additionally, anti-AGE and anti-MAA have been found to be associated with ESR in previous studies in RA and SLE.[22] PTMs and anti-PTMs such as anti-AGE and anti-MAA add to the understanding that the combined presence of the antigen and the antibody could trigger effector mechanisms and contribute to the overall process of arthritis and joint damage, in RA and also in non-RA. It would therefore be interesting to investigate whether next to carbamylated proteins[27] also the modifications AGE and MAA are present in cartilage and synovium. Additionally, experimental pathogenicity studies on anti-AGE and anti-MAA specifically should be performed to elucidate on the contribution of these anti-PTM antibodies to pathogenesis.

There are some limitations to our study. Data on anti-CarP antibody levels were missing for 149 patients with RA; therefore, analysis using stratification including anti-CarP could only be performed in a subgroup of all patients with RA. However, this group still consists of 499 patients with RA and therefore still appears a good representation of the RA population. Radiological progression was assessed in 635 patients with RA included before 2006. Thereafter, radiographs have not been scored since radiographic damage has become rare/nearly non-existent with current treatment strategies. This effectively enabled us to detect differences in the, earlier, informative part of the cohort. When stratifying radiological progression data, groups became small and therefore could suffer from insufficient power implicating that significance could not always be reached. It is therefore important to verify associations using different and/or bigger cohorts to be able to generalise findings to the whole RA population. Additionally, in order to verify the results obtained in this study, a replication cohort is needed. In such a study, IgA and IgM responses could be included to elaborate on the full anti-PTM antibody responses in patients with (rheumatoid) arthritis.[28 29] One of the strengths of this study is that the EAC is a well-defined cohort containing RA and non-RA early arthritis patients with extensive information on the HLA haplotype and radiological progression for patients with RA.[15] Second, antibody responses have been investigated on the PTM-modified proteins and their control proteins. All PTMs were created on the same antigen backbone and reactivity against FCS itself was subtracted from the results. This results in reliable measurements that capture truly PTM-specific signals and decreases the chance of false observations.[30] Additionally, correlation analyses were performed (data not shown) and data were stratified for the other investigated anti-PTM and to verify that anti-AGE and anti-MAA are solely responsible for the observed result and not cross-reactive.

In conclusion, anti-AGE and anti-MAA antibodies are both prevalent in patients with RA, and other inflammatory rheumatic conditions, and although not specific for RA they each correlate with specific parameters. Anti-MAA associates with HLA-DRB1*03 in CCP2-negative (RA) patients independent of anti-CarP and associates with inflammation. Anti-AGE associates with HLA-DRB1*03 in patients with CCP2-negative RA and is associated with a worse radiological progression especially in patients with anti-CCP2-negative and anti-CarP-negative RA. With this study, we have now characterised a seropositive subgroup within the heterogeneous group of patients with RA that have been thus far been considered seronegative.

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**Collaborators** not applicable.

**Contributors** All authors were involved in the design and interpretation of the study. Anti-PTM antibody analyses and interpretation were performed by MDvdB and NVB under the supervision of LAT. Statistical analyses on EAC cohort data were performed by TJvW and ATWH under the supervision of DvdW. Remission analysis on EAC cohort data was performed by AHMvdHvM and DvdW. LAT is the guarantor for this study.

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**Competing interests** TWJH, REMT and LAT are listed as inventors on a patent describing the methods to detect anti-CarP antibodies.

**Patient consent for publication** Not applicable.

**Ethics approval** The Leiden Early Arthritis Clinic (EAC) cohort was approved by the Medical Ethics Committee Leiden The Hague Delft under reference number: B19.008. For the current study, measurement of anti-PTM antibodies in serum from the EAC cohort is approved under reference number B15.003. Participants gave informed consent to participate in the study before taking part.

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**Data availability statement** Data are available upon reasonable request. Requests can be sent to l.a.trouw@lumc.nl.



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