# Cross-reactivity of anti-modified protein antibodies is also present in predisease and individuals without rheumatoid arthritis

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The presence of anti-citrullinated protein antibodies (ACPAs), anti-carbamylated protein antibodies (anti-CarPAs) and anti-acetylated protein antibodies (AAPAs) is a hallmark of rheumatoid arthritis (RA). ACPA and anti-CarPA can already be detected years before RA onset.[1] Moreover, it has been shown that the citrullinated epitope recognition profile of ACPA expands before RA develops. Recently, it has become clear that ACPA can display cross-reactivity to other post-translational modifications (PTMs), more specifically homocitrulline and

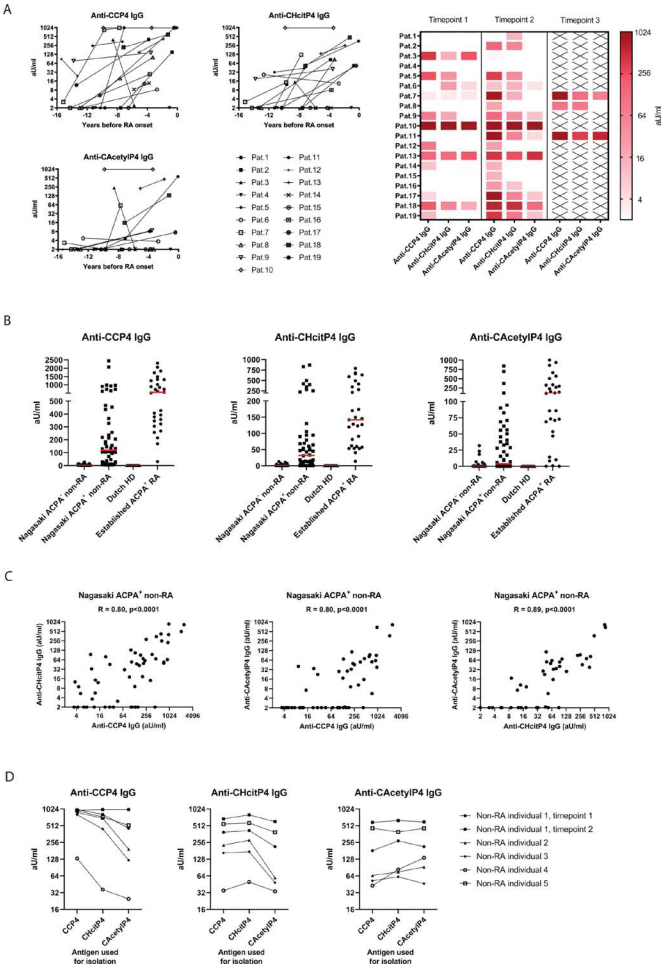


**Figure 1** (A) ACPA, anti-CarP and AAPA IgG levels, using the CCP4, CHcitP4 and CAcetylP4 peptides as antigen, in arbitrary units per ml (aU/mL) over time of 19 patients with RA before disease onset. Left graphs show the data in years before onset. The heatmap on the right shows a summary of the AMPA IgG levels per time point. (B) ACPA, anti-CarP and AAPA IgG levels in aU/ml of Japanese ACPA-non-RA samples (n=197), Japanese AcpA +non RA samples (n=54), Dutch healthy donors (n=30) and established patients with RA (n=29). (C) Correlations of ACPA, anti-CarP and AAPA levels in aU/mL in Japanese ACPA +non RA samples. R=correlation coefficient. (D) ACPA, anti-CarP and AAPA IgG levels in aU/mL of six samples from Japanese AcpA +non RA samples after antibody isolation using CCP4, CHcitP4 or CAcetylP4 peptides. ACPA, anticitrullinated protein antibody; AAPA, antiacetylated protein antibody; anti-CarPA, anti-carbamylated protein antibodies; RA, rheumatoid arthritis.

acetyllysine, as shown at both the monoclonal and polyclonal antibody level.[2 3] B cell receptor analysis of ACPA-expressing B cells from patients with RA has shown that ACPAs have undergone extensive somatic hypermutation and that this can facilitate epitope spreading to multiple citrullinated epitopes.[4] Given the association of ACPA epitope spreading with progression to disease, it is relevant to obtain more insights when cross-reactivity to other PTMs is introduced. Furthermore, insights in whether cross-reactivity is also present in ACPA-positive subjects without RA or confined to subjects that will—or have developed RA will also help to better understand the evolution of anti-modified protein antibody (AMPA) responses. Therefore, we analysed cross-reactivity of the ACPA response in pre-disease samples and ACPA-positive individuals without RA. To this end, ACPA, anti-CarPA and AAPA in different cohorts were measured using modified peptides as described in online supplemental materials. First, we analysed the AMPA-IgG response in samples from 19 different Swedish subjects who later developed RA. As expected, ACPA could be detected years before disease onset with a rise in antibody level over time (figure 1A). We detected a similar pattern for anti-CarPA and AAPA. Interestingly, for most patients with detectable ACPA, anti-CarPA and/or AAPA, these antibodies could be detected at the same timepoint, indicating their simultaneous appearance years before disease onset. Next, we analysed AMPA levels in samples from ACPA-positive and ACPA-negative Japanese individuals without RA, derived from the community-based Nagasaki Island study (figure 1B, online supplemental figure S1).[5] Intriguingly, a strong correlation between levels of the different individual AMPA-reactivities was observed, pointing to cross-reactivity of the antibodies (figure 1C). To experimentally confirm cross-reactivity, we selected six samples from ACPA-positive non-RA individuals with high AMPA values, isolated ACPA, anti-CarPA and AAPA and determined the reactivity of the isolated antibodies to the three different PTMs. Isolated ACPAs were highly reactive to the homocitrullinated and acetylated antigen and vice versa, showing that AMPA in individuals without RA are also cross-reactive towards different PTMs (figure 1D). These results were confirmed on post-translationally modified fibrinogen and FCS (online supplemental figure S2). Interestingly, the reactivity to citrullinated/homocitrullinated peptides was higher when AMPA were isolated with a citrullinated or homocitrullinated antigen than with an acetylated antigen. This suggests cross-reactivity between ACPA and anti-CarPA is stronger than between either of them and AAPA. Together, our data show that ACPA, anti-CarPA and AAPA already coexist before disease onset. Moreover, ACPA can be cross-reactive towards homocitrulline and acetyllysine in ACPA-positive individuals without RA. These results indicate that cross-reactivity towards different PTMs emerges when AMPA responses become detectable and provide evidence that cross-reactivity towards different PTMs is an intrinsic characteristic of AMPA responses. This finding is in line with the observation that (germline) ACPA-IgM can be cross-reactive towards other PTMs as well.[6] Although cross-reactivity seems to be an intrinsic feature of AMPA, it is tempting to speculate that the most cross-reactive B cells are selected during progression towards RA, explaining the increase of the ACPA epitope recognition profile in time towards disease onset. Although cross-reactivity is already present before disease onset, the further increase in AMPA cross-reactivity and level could be a valuable biomarker in predicting transition towards disease.

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