Title: Anti-CCP3.1 and Anti-CCP-IgA Are Associated with Increasing Age in Individuals Without Rheumatoid Arthritis

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ABSTRACT

Objectives: We investigated the association of age and anti-cyclic citrullinated peptide (CCP) antibodies in subjects without RA.

Methods: Serum was tested for anti-CCP3.1 (IgG/IgA) in 678 first-degree relatives (FDRs) of RA patients and 330 osteoarthritis (OA) subjects. Individual isotypes (anti-CCP-IgA and anti-CCP-IgG) were also tested in all FDRs.

Results: In FDRs, increasing age was significantly associated with positivity for anti-CCP3.1 (per year, OR=1.03) and anti-CCP-IgA (per year, OR=1.05) but not anti-CCP-IgG. In FDRs and OA subjects, anti-CCP3.1 prevalence was significantly increased after age 50.

Conclusions: Increasing age in individuals without RA should be considered in the interpretation of anti-CCP3.1 positivity.

INTRODUCTION

Antibodies to citrullinated protein/peptide antigens (ACPA) play an important role in rheumatoid arthritis (RA) pathogenesis and can be detected several years before the onset of inflammatory arthritis (IA) during a preclinical period of autoimmunity(1, 2). Understanding how ACPA develop in individuals without RA is informative to better delineate RA pathogenesis. Commercial ACPA assays for anti-cyclic citrullinated peptide (CCP) detect IgG-ACPA only (CCP2, CCP3) or both IgG-ACPA and IgA-ACPA with the CCP3.1 assay that was developed to add sensitivity in early RA diagnosis(3). While IgG-ACPA and IgA-ACPA are both specific for predicting future classifiable RA(4), they associate with different RA features suggesting different ACPA isotypes can be associated with different aspects of RA pathogenesis(5).

When considering factors that could influence ACPA, age is of interest because several autoantibodies are associated with increasing age(6-9), and ACPA positivity is higher in older RA-free women(10). However, the influence of age on isotype-specific ACPA has not been thoroughly investigated. Therefore, we evaluated the relationship of IgA-ACPA and IgG-ACPA with age in subjects without RA.

METHODS

Study Subjects

In this cross-sectional study, we included stored serum from two groups: 1) first-degree relatives (FDRs) of RA patients and 2) subjects with osteoarthritis (OA). Both cohorts had

no history of clinically-evident IA, therefore meeting no definable criteria for RA. For FDRs, 678 were randomly selected from the Studies of the Etiology of RA (SERA) cohort that includes a total of 1754 FDRs without a history of IA(11). Demographic and smoking history was obtained by questionnaire. For the OA cohort, 330 subjects were included who were previously recruited from Veterans Affairs Medical Centers and one academic medical center as a control group in a study examining environmental risk factors for RA(12). Medical record review was performed to obtain demographics and smoking history and to exclude the presence of IA. Family history of RA was not obtained.

Ethics: All study related procedures were approved by the Institutional Review Boards at each site (COMIRB #01-675). Informed written consent was obtained for all subjects for publication of data.

Autoantibody, Total Immunoglobulin (Ig) and Shared Epitope Testing

In all subjects, serum was tested for ACPA using commercial CCP3.1 ELISA (IgG/IgA, Inova Diagnostics Inc., San Diego, CA, USA). All FDRs were also tested for isotype-specific anti-CCP-IgA and anti-CCP-IgG using a CCP3 plate with the respective secondary antibody conjugate (Inova, for research only). For anti-CCP-IgA and anti-CCP-IgG, an in-house standard curve was generated from pooled RA subject serum. Cut-off levels for IgA-ACPA and IgG-ACPA positivity were set at the 98th percentile in a separate set of 126 healthy controls (no FDR with RA, mean age 36 years, 81% female).

Additional serum testing had previously been completed on FDRs as part of the SERA study(11) including testing for CCP2 (IgG, Diastat, Axis-Shield Diagnostics, Ltd., Dundee, United Kingdom) and RF by nephelometry (Dade-Behring) in all FDRs, for anti-CCP3 (IgG, Inova) in a random selection of 338 of the 678 FDRs and for total IgA and IgG (Beckman-Coulter Synchron nephelometry system) in 108 FDRs who had previously participated in lung-related SERA studies(13). All OA subjects were also tested for anti-CCP3 (IgG, Inova). Positivity for all commercial CCP assays was based on manufacturer recommendations. RF cut-off levels for positivity were set at the 95th percentile in 491 anonymous blood donors. Subjects were also tested for presence of shared epitope alleles using previously described methodologies(11).

Statistical Analysis

Univariate and multivariate logistic regression was used to determine associations between age as a continuous variable in years and ACPA positivity (See Supplemental Methods). We also compared prevalence of ACPA positivity between age groups as a categorical variable (by decade) using Chi-square/Fisher's exact testing, with the youngest decade (18-29 years) serving as the referent group. We used Cohen's kappa to compare agreement between assays, McNemar's test to compare anti-CCP positivity within groups and Pearson's correlation to compare age and total Ig levels. Analyses were completed using SPSS24 (IBM, Armonk, NY, USA) and SAS9.4 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Anti-CCP3.1 and Age in FDRs

Subject demographics are in Table 1. In FDRs, 62/678 (9.1%) were anti-CCP3.1 positive (Table 1). There was a significant association between anti-CCP3.1 positivity and increasing age (OR=1.04, 95% CI 1.02-1.05) that remained significant in the adjusted model (Table 2). Compared to the 18-29 year old referent group, anti-CCP3.1 positivity was more prevalent in all age groups ≥50 years (i.e. 50-59, 60-69 and ≥70 years) (Figure 1, Panel A; Supplemental Table 2). To account for the low prevalence of positivity in 18-29 year olds, we also performed comparisons using a larger referent group of 18-39 year old FDRs and found similar results (Supplemental Table 3). When stratified by sex, anti-CCP3.1 was associated with age in both women (1.03, 95% CI 1.01-1.05) and men (OR=1.07, 95% CI 1.02-1.11).

IgA-ACPA, IgG-ACPA and Age in FDRs

In FDRs, 58/678 (8.6%) were anti-CCP-IgA positive and 33/678 (4.9%) were anti-CCP-IgG positive. Similar to anti-CCP3.1, anti-CCP-IgA positivity was associated with increasing age overall (OR=1.04, 95% CI 1.03-1.06; Table 2) and in both women (OR=1.04, 95% CI 1.02-1.06) and men (OR=1.09, 95% CI 1.04-1.15). Compared to the referent group, anti-CCP-IgA was more prevalent in FDRs in all age groups ≥40 years (i.e. 40-49, 50-59, 60-69 and ≥70 years) (Figure 1, Panel B; Supplemental Table 2). There was no association between anti-CCP-IgG and age (Table 2). There was good agreement between anti-CCP3.1 and anti-CCP-IgA or anti-CCP-IgG positivity (kappa=0.68).

Anti-CCP2, Anti-CCP3, RF and Total Igs in FDRs

Using commercial CCP assays that detect only IgG reactivity, 19/678 (2.8%) FDRs were anti-CCP2 positive and 19/388 (4.9%) were anti-CCP3 positive. There was no association between age and anti-CCP2 or anti-CCP3 positivity in FDRs (p=0.63 and p=0.43, respectively). In addition, 41/678 (6.0%) FDRs were RF positive, and again with no association between age and positivity (p=0.26). There was also no association between age and level of total IgG or total IgA (p=0.27 and p=0.42, respectively).

Anti-CCP in OA Subjects

To investigate whether the association of anti-CCP3.1 and age was specific to FDRs, we studied a separate cohort of older subjects with OA. There was a trend toward an association between anti-CCP3.1 positivity and increasing age in OA subjects (OR=1.04, 95% CI 1.0-1.07).

Furthermore, anti-CCP3.1 was more prevalent than anti-CCP3 positivity in OA subjects ≥50 years (25/275 (9.1%) vs. 11/275 (4.0%), p<0.01), but not in OA subjects <50 years (3/55 (5.5%) vs. 3/55 (5.5%), p=1.0). In addition, FDRs ≥50 years were more likely than OA subjects ≥50 years to be anti-CCP3.1 positive (41/271 (15.1%) vs. 25/275 (9.1%), p=0.03).

DISCUSSION

We identified a significant association between anti-CCP3.1 positivity and increasing age in subjects without RA, which appears to be driven by anti-CCP-IgA. While both FDRs

and OA subjects ≥50 years had increased anti-CCP3.1 positivity, this association was stronger in FDRs suggesting that other familial or shared environmental components may contribute to ACPA development in older subjects. In contrast to prior reports(10, 14), we identified a significant association between anti-CCP3.1 and age in both women and men suggesting that the higher prevalence of ACPA in older individuals may be more generally related to aging rather than a hormonal effect specific to women. While our study focused on subjects without RA, we did not identify a similar association between anti-CCP3.1 and increasing age in SERA subjects with established RA (data not shown). Although, it could be that this relationship is masked by the high prevalence of anti-CCP3.1 positivity in RA. It is of interest that an association between anti-CCP secretory IgA and increasing age has been described in subjects with early classified RA(15), although this study did not specifically exclude the possibility of anti-CCP secretory IgM(16).

Several features of aging could potentially contribute to ACPA development including the accumulation of oxidative post-translational protein modifications and increased self-reactive IgA that are not always associated with tissue injury(7). Older individuals also have increased dysfunctional terminally differentiated B-cells hypothesized to secrete antibodies in response to chronic antigen stimulation by persistent viruses or autoantigens(17). Autoantibodies in older individuals may also have regulatory functions such as targeting senescent cells for clearance to maintain homeostasis and balance the wear and tear of aging(18).

Regarding the clinical impact of our findings, our data highlight the importance of considering age in the interpretation of anti-CCP3.1 in subjects without IA. While not all clinical laboratories use CCP3.1, some of the largest reference laboratoris (e.g. LabCorp) currently uses CCP3.1, supporting clinical relevance of our findings. Furthermore, because assay specificity is based on performance within a disease-free population, the specificity of anti-CCP3.1 for RA would be decreased in older individuals. In addition, elderly onset RA may differ in antibody profiles compared to those with younger onset. Regarding the impact of our findings on RA pathogenesis, our findings align with the hypothesis that early ACPA generation may begin at an IgA-predominant mucosal site(13, 19, 20). However, auto-reactive IgA may also be less pathogenic than IgG(7), and IgA-ACPA alone may be less predictive of developing classified RA(4).

Caveats to our study include the cross-sectional design and lack of RA family history in OA subjects that would be needed to confirm that age was responsible for the increased ACPA prevalence independent of RA family history. Furthermore, because our OA cohort was chosen based on their older mean age, it included a limited number of younger individuals for comparison. In addition, because IgG-ACPA is more predictive of developing RA(1), IgG-ACPA positive non-RA subjects may more rapidly develop classified RA resulting in lower IgG-ACPA positivity in older non-RA cohorts. As expected, we found good agreement between anti-CCP3.1 (IgG/IgA) positivity and the individual isotypes anti-CCP-IgA or anti-CCP-IgG positivity. We assume that the increased anti-CCP3.1 positivity in OA subjects ≥50 years was driven by IgA-ACPA, but individual IgA-ACPA testing should be performed to confirm. Future studies are also needed to evaluate

associations of age and secretory IgA-ACPA, to determine specific epitopes targeted by ACPA in older individuals and to establish the performance of anti-CCP3.1 positivity to predict future classified RA in older individuals.

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Figure 1. Anti-CCP positivity and levels by age group in SERA FDRs. Panel A displays the percentage of FDRs with anti-CCP3.1 positivity at standard cut-off levels (black bars) and at 3x the standard cut-off level (gray bars) by age group. Panel B displays the percentage of FDRs with anti-CCP3.1 positivity (black bars), anti-CCP-IgA positivity (gray bars) and anti-CCP-IgG positivity (white bars) by age group. Panel C displays the median level (IQR) for anti-CCP-IgA (black bars) and anti-CCP-IgG (gray bars) by age group. *p<0.05, **p<0.01 comparing each outcome to the same outcome in the 18-29 year old reference age group. The total number of FDRs included in each age group includes: N=115 for 18-29 years, N=138 for 30-39 years, N=154 for 40-49 years, N=131 for 50-59 years, N=82 for 60-69 years and N=58 for ≥70 years.

Table 1. Subject characteristics[1]

	FDRs (n=678)	OA (n=330)	P value
Age, mean years (SD)	46 (16)	60 (11)	<0.01
Female	505 (75)	132 (40)	<0.01
Non-Hispanic White	515 (76)	239 (72)	0.23
Ever Smoker	267 (40)	153 (46)	0.04
Current Smoker	75 (11)	36 (11)	0.94
≥1 shared epitope allele	351 (52)	130 (45)	0.05
Body mass index, kg/m^2, mean (SD)	27.4 (5.9)	-	-
Education, > high school	533 (79)	-	-
Anti-CCP3.1 positive	62 (9)	28 (9)	0.73

1. All values are reported as N(%) unless otherwise specified. Missing values: Shared epitope, n=4 FDRs, n=42 OA subjects; Smoking, n=2 FDRs; Race/ethnicity, n=4 FDRs; BMI, n=12 FDRs

Table 2. Associations of age and ACPA positivity in SERA FDRs

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Anti-CCP3.1	1.04 (1.02, 1.05)	1.03 (1.02, 1.05)[1]
Anti-CCP-IgA	1.04 (1.03, 1.06)	1.05 (1.03, 1.06)[2]
Anti-CCP-IgG	1.01 (0.99, 1.03)	1.004 (0.98, 1.03)[3]

1. Adjusted for body mass index (BMI) and rheumatoid factor (RF)

2. Adjusted for BMI, sex and education

3. Adjusted for RF and education

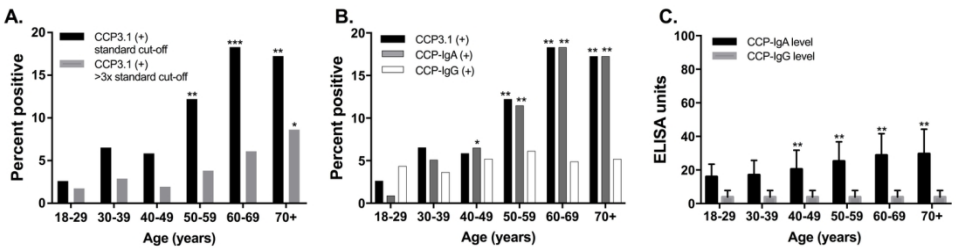

Figure 1. Anti-CCP positivity and levels by age group in SERA FDRs. Panel A dis-plays the percentage of FDRs with anti-CCP3.1 positivity at standard cut-off levels (black bars) and at 3x the standard cut-off level (gray bars) by age group. Panel B dis-plays the percentage of FDRs with anti-CCP3.1 positivity (black bars), anti-CCP-IgA positivity (gray bars) and anti-CCP-IgG positivity (white bars) by age group. Panel C displays the median level (IQR) for anti-CCP-IgA (black bars) and anti-CCP-IgG (gray bars) by age group. *p<0.05, **p<0.01 comparing each outcome to the same outcome in the 18-29 year old reference age group. The total number of FDRs included in each age group includes: N=115 for 18-29 years, N=138 for 30-39 years, N=154 for 40-49 years, N=131 for 50-59 years, N=82 for 60-69 years and N=58 for ≥70 years.

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