EXTENDED REPORT

# Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles

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**Objectives:** To study the gene–environment interaction of tobacco exposure and shared epitope on autoantibodies in patients with rheumatoid arthritis and undifferentiated arthritis.
**Methods:** From incident cases of arthritis (n = 1305), patients who did not fulfil any classification criteria (undifferentiated arthritis (n = 486)) and those who fulfilled the American College of Rheumatology criteria for rheumatoid arthritis (n = 407) were identified. IgM rheumatoid factor (RF), anti-cyclic-citrullinated peptide (CCP) antibodies, and HLA-DRB1 alleles were determined.
**Results:** In rheumatoid arthritis, an interaction was found between tobacco exposure and shared epitope for the presence of anti-CCP antibodies, as the odds ratio for anti-CCP antibodies in patients having both tobacco exposure (TE) and shared epitope (SE) was higher than the summed odds ratios of patients having only tobacco exposure or shared epitope (odds ratios: TE+/SE−, 1.07; TE−/SE+, 2.49; and TE+/SE+, 5.27—all relative to TE−/SE−). A similar effect was found for RF, but stratification showed that the interaction primarily associated with the anti-CCP antibody response. In patients with undifferentiated arthritis at two weeks, or with persistent undifferentiated arthritis after one year, no interaction between tobacco exposure and shared epitope was observed for the presence of autoantibodies.
**Conclusions:** Tobacco exposure increases the risk factor for anti-CCP antibodies only in shared epitope positive patients with rheumatoid arthritis. The gene–environment interaction between smoking and shared epitope leading to autoantibodies is specific for rheumatoid arthritis and is not observed in undifferentiated arthritis.

In the search for the aetiology of rheumatoid arthritis, genetic predisposition, environmental risk factors, and dietary risk factors may provide clues to the pathogenesis.[1–7] The most important genetic risk factor for rheumatoid arthritis is the presence of HLA class II alleles which share the conserved amino acid sequence called the shared epitope.[8] These shared epitope residues constitute a part of the antigen-presenting binding site. The shared epitope hypothesis postulates that the shared epitope motif itself is directly involved in the pathogenesis of rheumatoid arthritis by allowing the presentation of a peptide to arthritogenic T cells. The most prominent environmental risk factor for rheumatoid arthritis is smoking; smokers have increased levels of rheumatoid factor (RF),[9–11] are more prone to develop rheumatoid arthritis,[11–14] and develop more severe disease.[15–17] Interaction between environmental and genetic risk factors points to the existence of disease specific pathogenic pathways involved in disease induction or progression.

For rheumatoid arthritis, Padyukov *et al* recently described a gene–environment interaction between smoking and shared epitope that provides risk (odds ratio (OR) = 2.8 (95% confidence interval (CI), 1.6 to 4.8)) for RF positive but not RF negative rheumatoid arthritis in a large cohort of 858 RF positive and 1048 RF negative patients with the disease.[18] Recently, studying two large cohorts from the USA and Europe using different genetic epidemiological methods (association and linkage), we showed that HLA-DRB1 alleles are only a risk factor for rheumatoid arthritis in people who have anti-cyclic-citrullinated peptide (CCP) antibodies and not in the absence of such antibodies, suggesting different pathogenic pathways for anti-CCP positive and anti-CCP negative rheumatoid arthritis.[19]

In the present study we investigated whether the gene–environment interaction smoking–shared epitope is also present for the anti-CCP antibody response, and whether this interaction was more pronounced for development of RF compared to development of the anti-CCP antibodies. Second, we aimed to assess whether the interaction of smoking and shared epitope is specific for patients with rheumatoid arthritis or is also present in undifferentiated arthritis. To this end patients with arthritis who did not fulfil any classification criteria at presentation and patients with persistent undifferentiated arthritis at a one year follow up were studied. Patients who present with undifferentiated arthritis have a spontaneous remission rate of about 50%[20] and might have differences in the underlying pathogenesis. If the interaction between smoking and shared epitope that leads to autoantibody formation is specific for the pathogenesis of rheumatoid arthritis, we hypothesised that such an interaction would not be seen in patients with undifferentiated arthritis who, on clinical follow up, have not developed rheumatoid arthritis.

**Abbreviations:** ACR, American College of Rheumatology; CCP, cyclic citrullinated peptide; EAC, early arthritis clinic; RF, rheumatoid factor; SE+/−, shared epitope positive/negative; TE+/−, tobacco exposure positive/negative

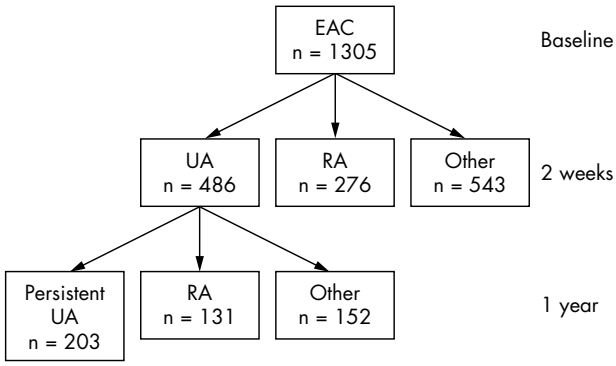


**Figure 1** Flow chart of early arthritis clinic patients. RA, rheumatoid arthritis; UA, undifferentiated arthritis.

## PATIENTS AND METHODS

### Patients

For this study, we used the Leiden early arthritis clinic (EAC), a population based inception cohort of patients with newly diagnosed early arthritis (for further information, see Van Aken *et al*[21]). Rheumatoid arthritis was diagnosed according to the American College of Rheumatology (ACR) criteria of 1987.[22] Patients who could not properly be classified according to one of the ACR criteria at a two week follow up were categorised as having undifferentiated arthritis.[20] This population of patients with undifferentiated arthritis was further divided as follows:

- those who had developed rheumatoid arthritis;
- those who remained unclassified (persistent undifferentiated arthritis);
- those who had developed other rheumatic diseases such as spondylarthropathies, osteoarthritis, gout, or reactive arthritis at a one year follow up (fig 1).

At inclusion in the EAC cohort, smoking status (cigarettes, cigars) was recorded as past, current, or never smoked for each subject. Current and past smokers were classified as tobacco exposure positive (TE+) and never smokers as tobacco exposure negative (TE−).[11] Baseline laboratory indices included C reactive protein, IgM RF (enzyme linked immunosorbent assay (ELISA), as previously described[23]), anti-CCP antibodies (ELISA, Immunoscan RA Mark 2, EuroDiagnostica, Arnhem, Netherlands and Axis-Shield, Dundee, UK) and HLA class II alleles. HLA-DRB1 (sub-)typing was undertaken by polymerase chain reaction using specific primers and hybridisation with sequence specific oligonucleotides. Shared epitope alleles were: DRB1 *0101, *0102, *0401, *0404, *0405, *0408, *0410, and *1001.[24] Patients homozygous and heterozygous for shared epitope were both classified as SE+. Missing data for the whole cohort of 1305 patients ranged between 0% and 20% for several items (shared epitope, tobacco exposure, anti-CCP, and RF). An analysis of the baseline values of patients with missing data points showed no differences from patients without missing data points.

### Statistical analysis

Odds ratios (OR) were calculated for the primary outcome measures RF and anti-CCP antibodies. Stratified analysis was undertaken for anti-CCP positive, anti-CCP negative, RF positive, and RF negative strata. We used $\chi^2$ analysis on 2×4 tables.

## RESULTS

### Patient characteristics

Between 1993 and 2003, 1305 patients were included in the EAC cohort. At the two weeks follow up, 486 patients did not fulfil any classification criteria and were thus classed as undifferentiated arthritis. At this time 276 patients fulfilled the ACR criteria for rheumatoid arthritis. Of the 486 patients with undifferentiated arthritis, after the one year follow up 131 patients were diagnosed as having rheumatoid arthritis. In 203 patients the diagnosis remained undifferentiated arthritis (persistent undifferentiated arthritis) and in the other 152 patients another rheumatic disorder—such as spondylarthropathy, osteoarthritis, or psoriatic arthritis—was identified (fig 1). The total number of patients from the cohort who were identified as having rheumatoid arthritis at one year was 407. From the patients classified as persistent undifferentiated arthritis at one year, 15% developed rheumatoid arthritis during further follow up (Verpoort K, unpublished data).

Baseline patient characteristics of the patients who presented with rheumatoid arthritis or undifferentiated arthritis at two weeks are given in table 1; the data on the patients with undifferentiated arthritis are presented both for those who developed rheumatoid arthritis after the one year follow up and for those who remain undifferentiated. Patients with undifferentiated arthritis who did not develop rheumatoid arthritis were younger at presentation than those who went on to develop rheumatoid arthritis (mean age 48

**Table 1** Patient characteristics at baseline of patients that presented with rheumatoid arthritis, patients that presented with undifferentiated arthritis and developed rheumatoid arthritis after 1 year and patients that presented with undifferentiated arthritis and had other diagnosis than rheumatoid arthritis after one year follow-up

| | RA at 2 weeks (n = 276) | UA→RA (n = 131) | UA→non-RA (n = 355) | p Value* |
|---|---|---|---|---|
| Age (years) (mean) | 58 | 56 | 48 | <0.001 |
| Female (%) | 66 | 64 | 51 | NS |
| CRP (mg/l) mean) | 35 | 29 | 21 | 0.036 |
| IgM RF+ (%) | 65 | 52 | 13 | <0.001 |
| Anti-CCP+ (%) | 54 | 51 | 7 | < 0.01 |
| SE+ (%) | 68 | 63 | 49 | 0.046 |
| TE+ (%) | 47 | 52 | 50 | NS |

*Determined for UA→RA *v* UA→non-RA. Comparison of RA at two weeks *v* UA→RA showed no significant differences.
Anti-CCP+, anti-cyclic-citrullinated peptide positive; CRP, C reactive protein; RA, rheumatoid arthritis; RF+, rheumatoid factor positive; SE+, presence of one or two shared epitope alleles; TE+, current and past smokers as indicated in the medical history; UA, undifferentiated arthritis.

**Table 2** Odds ratios for developing rheumatoid factor and anti-cyclic-citrullinated peptide antibodies in the presence of tobacco exposure and/or shared epitope alleles in all patients with rheumatoid arthritis at one year

| | TE | SE | RF+ | RF− | OR | 95% CI | p Value |
|---|---|---|---|---|---|---|---|
| | − | − | 23 | 31 | 1.00 | – | – |
| | + | − | 25 | 23 | 1.47 | 0.62 to 3.45 | 0.33 |
| | − | + | 54 | 54 | 1.35 | 0.66 to 2.75 | 0.37* |
| | + | + | 72 | 30 | 3.23 | 1.54 to 6.81 | <0.001* |
| | | | | | | | 0.002 π |
| | **TE** | **SE** | **Anti-CCP+** | **Anti-CCP−** | **OR** | | **p Value** |
| | − | − | 18 | 34 | 1.00 | − | – |
| | + | − | 17 | 30 | 1.07 | 0.43 to 2.65 | 0.87 |
| | − | + | 58 | 44 | 2.49 | 1.18 to 5.31 | 0.01† |
| | + | + | 67 | 24 | 5.27 | 2.37 to 11.80 | <0.001† |
| | | | | | | | <0.001 π |
| **Anti-CCP** | **TE** | **SE** | **RF+** | **RF−** | **OR** | **95% CI** | **p Value** |
| + | − | − | 14 | 4 | 1.00 | – | – |
| + | + | − | 17 | 0 | ∞ | ∞ | 0.10 |
| + | − | + | 46 | 12 | 1.10 | 0.22 to 4.42 | 0.88‡ |
| + | + | + | 54 | 13 | 1.19 | 0.24 to 4.68 | 0.79‡ |
| | | | | | | | 0.23 |
| − | − | − | 7 | 27 | 1.00 | – | – |
| − | + | − | 8 | 22 | 1.40 | 0.38 to 5.32 | 0.57 |
| − | − | + | 6 | 38 | 0.61 | 0.12 to 2.40 | 0.41‡ |
| − | + | + | 7 | 17 | 1.59 | 0.39 to 6.34 | 0.45‡ |
| | | | | | | | 0.39π |
| **RF** | **TE** | **SE** | **Anti-CCP+** | **Anti-CCP−** | **OR** | **95% CI** | **p Value** |
| + | − | − | 14 | 7 | 1.00 | – | – |
| + | + | − | 17 | 8 | 1.06 | 0.26 to 4.34 | 0.92 |
| + | − | + | 46 | 6 | 3.83 | 0.91 to 16.07 | 0.03‡ |
| + | + | + | 54 | 7 | 3.86 | 0.96 to 15.11 | 0.02‡ |
| | | | | | | | 0.02π |
| − | − | − | 4 | 27 | 1.00 | – | – |
| − | + | − | 1 | 22 | 0.31 | 0.01 to 3.47 | 0.28 |
| − | − | + | 12 | 38 | 2.13 | 0.56 to 9.97 | 0.22† |
| − | + | + | 13 | 17 | 5.16 | 1.28 to 24.71 | 0.01‡ |
| | | | | | | | 0.04π |

*TE−/SE+ *v* TE+/SE+: OR = 2.4 (95% CI, 1.3 to 4.4), p = 0.002.
†TE−/SE+ *v* TE+/SE+: OR = 2.1 (95% CI, 1.1 to 4.1), p = 0.02).
‡Comparison of TE−/SE+ *v* TE+/SE+ not significant.
π, p value of $\chi^2$ analysis of 2×4 table.
CI, confidence interval; anti-CCP, anti-cyclic-citrullinated peptide; OR, odds ratio; RF, rheumatoid factor; SE, presence of one or two shared epitope alleles; TE, current and past smokers as indicated in the medical history.

years *v* 56 years, p<0.001). The patients with undifferentiated arthritis who developed rheumatoid arthritis after one year had higher levels of C reactive protein, RF, and anti-CCP antibodies at baseline and were more often SE+ compared with those who had persistent undifferentiated arthritis or developed other rheumatological diagnoses (table 1). No differences were observed between the 131 patients who were diagnosed as having rheumatoid arthritis after one year and the 276 patients who were diagnosed at the two weeks visit.

## Interaction of tobacco exposure and shared epitope in rheumatoid arthritis

We analysed the interaction between shared epitope and tobacco exposure in patients with rheumatoid arthritis and undifferentiated arthritis. Outcome variables were RF and anti-CCP antibodies.

No effect of tobacco exposure on RF status was seen in any of the SE− patients with rheumatoid arthritis, in contrast to a clear effect in SE+ patients. The odds ratio for positive RF was 1.47 for TE+/SE− patients, 1.35 for TE−/SE+ patients, and 3.23 for TE+/SE+ patients, all relative to TE−/SE− patients (table 2), showing an interaction between tobacco exposure and shared epitope for the development of RF.

In shared epitope negative patients with rheumatoid arthritis, no effect of tobacco exposure was seen for positive anti-CCP antibodies, in contrast to a clear effect in the shared epitope positive group. The odds ratio for positive anti-CCP antibodies was 1.07 for TE+/SE− patients, 2.49 for TE−/SE+ patients, and 5.27 for TE+/SE+ patients, again all relative to TE−/SE− patients. As the odds ratio for anti-CCP antibodies of patients having both tobacco exposure and shared epitope was higher than the summed odds ratios of patients with only tobacco exposure or shared epitope, an interaction was found between tobacco exposure and shared epitope for the presence of anti-CCP antibodies. The difference between the TE−/SE+ patients and the TE+/SE+ patients was significant for both IgM-RF and anti-CCP (table 2).

Presuming that RF positive and anti-CCP positive patients partly overlap each other, a stratified analysis was carried out for RF positive and RF negative and anti-CCP positive and anti-CCP negative patients. The results (table 2) show that when stratified for the presence or absence of anti-CCP antibodies, no significant interaction was found between tobacco exposure and shared epitope in relation to the presence of RF. When stratified for RF, in the RF negative group an interaction between tobacco exposure and shared epitope was observed for the development of anti-CCP antibodies. These data suggest that the interaction between

**Table 3** Odds ratios for developing RF and anti-CCP antibodies in the presence of tobacco exposure and/or shared epitope alleles in patients with undifferentiated arthritis

| (A) All UA patients at two weeks: TE | SE | IgM RF+ | IgM RF− | OR | 95% CI | p Value |
|---|---|---|---|---|---|---|
| − | − | 12 | 55 | 1.00 | − | − |
| + | − | 13 | 48 | 1.24 | 0.47 to 3.29 | 0.63 |
| − | + | 19 | 60 | 1.45 | 4.60 to 3.59 | 0.34‡ |
| + | + | 18 | 54 | 1.53 | 0.62 to 3.82 | 0.31‡ |
| | | | | | | 0.75π |
| **TE** | **SE** | **Anti-CCP+** | **Anti-CCP−** | **OR** | **95% CI** | **p Value** |
| − | − | 6 | 54 | 1.00 | – | to |
| + | − | 9 | 48 | 1.69 | 0.49 to 6.19 | 0.35 |
| − | + | 20 | 47 | 3.83 | 1.33 to 12.53 | 0.01‡ |
| + | + | 21 | 47 | 4.02 | 1.40 to 13.09 | 0.01‡ |
| | | | | | | <0.01π |
| **(B) Subgroup of UA patients who develop RA within one year: TE** | **SE** | **IgM RF+** | **IgM RF−** | **OR** | **95% CI** | **p Value** |
| − | − | 6 | 12 | 1.00 | | |
| + | − | 8 | 14 | 1.14 | 0.26 to 5.24 | 0.84 |
| − | + | 15 | 22 | 1.36 | 0.37 to 5.44 | 0.61‡ |
| + | + | 19 | 13 | 2.92 | 0.76 to 11.90 | 0.08‡ |
| | | | | | | 0.87π |
| **TE** | **SE** | **Anti-CCP+** | **Anti-CCP−** | **OR** | **95% CI** | **p Value** |
| − | − | 8 | 10 | 1.00 | | |
| + | − | 8 | 13 | 0.77 | 0.18 to 3.33 | 0.69 |
| − | + | 19 | 16 | 1.48 | 0.41 to 5.46 | 0.50‡ |
| + | + | 21 | 9 | 2.92 | 0.74 to 11.66 | 0.08‡ |
| | | | | | | 0.12π |
| **(C) Subgroup of persistent UA patients at one year: TE** | **SE** | **IgM RF+** | **IgM RF−** | **OR** | **95% CI** | **p Value** |
| − | − | 6 | 37 | 1.00 | | |
| + | − | 5 | 32 | 0.96 | 0.21 to 4.20 | 0.95 |
| − | + | 9 | 34 | 1.63 | 0.46 to 6.17 | 0.39‡ |
| + | + | 4 | 33 | 0.75 | 0.14 to 3.48 | 0.67‡ |
| | | | | | | 0.62π |
| **TE** | **SE** | **Anti-CCP+** | **Anti-CCP−** | **OR** | **95% CI** | **p Value** |
| − | − | 1 | 36 | 1.00 | | |
| + | − | 2 | 34 | 2.12 | 0.10 to 128 | 0.54 |
| − | + | 8 | 27 | 10.67 | 1.26 to 486 | 0.01‡ |
| + | + | 4 | 32 | 4.50 | 0.41 to 227 | 0.16‡ |
| | | | | | | 0.03π |

‡Comparison TE−/SE+ *v* TE+/SE+ not significant.
π, p value of $\chi^2$ analysis of 2×4 table.
CI, confidence interval; anti-CCP, anti-cyclic-citrullinated peptide; OR, odds ratio; RF, rheumatoid factor; SE, presence of one or two shared epitope alleles; TE, current and past smokers as indicated in the medical history; UA, undifferentiated arthritis.

tobacco exposure and shared epitope primarily associates with positive anti-CCP antibodies and not with positive RF.

### Interaction of tobacco exposure and shared epitope in undifferentiated arthritis

In the whole group of patients who presented with undifferentiated arthritis, the combination of tobacco exposure and shared epitope did not significantly increase the risk for the presence of positive RF or anti-CCP antibodies (table 3). The odds ratio for positive anti-CCP antibodies in TE−/SE+ patients with undifferentiated arthritis was increased compared with TE−/SE− patients (OR = 3.83 (95% CI, 1.33 to 12.53)), but addition of tobacco exposure to the presence of shared epitope did not increase the risk of having anti-CCP antibodies (OR = 4.02 *v* 3.83, table 3A).

Subsequently we assessed whether in the subgroup patients with undifferentiated arthritis who developed rheumatoid arthritis (n = 131), smoking combined with the presence of shared epitope increased the risk of having anti-CCP antibodies. Thus the non-smoking SE− patients with undifferentiated arthritis who developed rheumatoid arthritis were compared with smoking SE− patients with undifferentiated arthritis who developed rheumatoid arthritis (OR = 0.77), as well as with non-smoking SE+ patients with undifferentiated arthritis who developed rheumatoid arthritis (OR = 1.48), and finally with smoking SE+ patients with undifferentiated arthritis who developed rheumatoid arthritis (OR = 2.92). In this smaller group of patients with rheumatoid arthritis, a trend for the interaction of shared epitope and tobacco exposure to increased risk of having anti-CCP antibodies was found. Calculations for RF outcome showed similar results (table 3B).

The same calculations were repeated for patients with persistent undifferentiated arthritis (n = 203). Although the number of anti-CCP positive patients with undifferentiated arthritis was small, no effect of tobacco exposure combined with shared epitope on the risk of having anti-CCP antibodies or RF was observed (table 3C). Thus the interaction of shared

epitope and tobacco exposure was found for the presence of RF and for anti-CCP antibodies in patients with rheumatoid arthritis and in those patients with undifferentiated arthritis who developed rheumatoid arthritis within one year but not in those with persistent undifferentiated arthritis.

## DISCUSSION

A strong gene–environment interaction between tobacco exposure and shared epitope for the presence of autoantibodies was observed. Intriguingly, this gene–environmental interaction was only present in patients with rheumatoid arthritis and was not observed in those with (persistent) undifferentiated arthritis. Stratified analysis for the different autoantibody responses (IgM RF and anti-CCP) showed that the interaction was primarily for the anti-CCP response.

A recent Swedish study found that a gene–environment interaction between shared epitope and smoking resulted in an increased risk specifically for RF positive rheumatoid arthritis.[18] These data were replicated and extended in the present study. Replication by a separate group in a separate cohort minimises the risk that the current findings are false positive.[25] Both the Swedish and the current data showed the lack of a relation between smoking and autoantibodies in shared epitope negative rheumatoid arthritis, indicating that this interaction is preferential for a given pathogenic pathway in shared epitope positive rheumatoid arthritis. To specify this pathogenic pathway with regard to the specificity of the autoantibody response, the stratified analysis for anti-CCP positivity yielded no additional effect of smoking on the risk of developing RF. In contrast, the stratified analysis for RF indicated that smoking more than doubled the risk in the shared epitope positive patients of developing anti-CCP antibodies. These data suggest that the gene–environment interaction between smoking and shared epitope leading to autoantibodies is primarily associated with the anti-CCP response. Apart from the specificity of this interaction, our group has recently found that shared epitope is a risk factor only for anti-CCP positive and not for anti-CCP negative rheumatoid arthritis.[19] The current data do not allow the analysis of smoking as a risk factor for anti-CCP positive *v* anti-CCP negative rheumatoid arthritis because no data of smoking in a matched group of the general population are known. However, given the fact that shared epitope alone is not a risk factor for anti-CCP negative rheumatoid arthritis,[19] the gene–environment interaction between smoking and shared epitope leading to anti-CCP antibodies seems characteristic for anti-CCP positive rheumatoid arthritis. Indeed no effect of smoking was observed in the shared epitope negative patients (table 2). These data are in line with our previously reported hypothesis that different pathogenic pathways operate in anti-CCP negative compared with anti-CCP positive rheumatoid arthritis. The demonstration of these pathogenic pathways is difficult because it is not known which proteins are citrinullated as a result of smoking, nor is it known if or how smoking destroys the normal tolerance to citrinullated self proteins.

In this study the diagnosis of persistent undifferentiated arthritis was defined as the presence of arthritis that did not fulfil any of the classification criteria after one year follow up. This may lead to some misclassification because a small proportion of patients with undifferentiated arthritis develop rheumatoid arthritis only after a longer time interval. However, in a previous analysis of this cohort this involved fewer than 15% of the patients with persistent undifferentiated arthritis at one year. More importantly, no interaction between smoking and shared epitope was observed at all in the persistent undifferentiated arthritis group.

A weakness of the current study is that the information on tobacco exposure was limited to patient history taking, and patients were not asked about the number of pack-years of smoking. Therefore no conclusions about the minimum exposure can be drawn.

In summary, smoking was confirmed to be a risk factor for positive anti-CCP and positive RF antibodies in the presence of shared epitope in patients with rheumatoid arthritis. In undifferentiated arthritis no interaction between tobacco exposure and shared epitope was demonstrated for the presence of autoantibodies.


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