

Original article

Autoantibodies to human citrullinated fibrinogen and their subfamilies to the α 36-50Cit and β 60-74Cit fibrin peptides similarly predict radiographic damages: a prospective study in the French ESPOIR cohort of very early arthritides

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Abstract

Objective. To investigate whether subfamilies of the RA-specific autoantibodies to human citrullinated fibrinogen (AhFibA) differentially associate with the RA risk factors, HLA-DRB1 shared epitope containing alleles (SE alleles) and cigarette smoking, and thus help to predict the disease outcome.

Methods. AhFibA and their anti- α 36-50Cit and anti- β 60-74Cit subfamilies were assayed by ELISA, at baseline, in the French ESPOIR (Etude et Suivi des Polyarthrites Indifférenciées Récentes) cohort composed of undifferentiated arthritides and RA patients of < 6 months' duration. Cigarette smoking, SE alleles' presence, DAS28, HAQ and modified Sharp–van der Heijde Score data were obtained at baseline, and after follow-up.

Results. After 3 years, 701 patients were classified as having RA according to the ACR/EULAR 2010 criteria. Among them, 349 (50%), 203 (29%) and 257 (37%) were AhFibA-, anti- α 36-50Cit- and anti- β 60-74Cit-positive, respectively. The presence and titres of AhFibA and their subfamilies similarly associated with SE alleles, irrespective of their fine specificity, without significant effect of smoking. Neither their presence nor their titre was associated with DAS28 or HAQ. The presence of at least one subfamily was associated with a faster Sharp/van der Heijde score progression, albeit without correlation with the titre.

Conclusion. AhFibA and their main subfamilies are similarly associated with SE alleles without additional effect of smoking. Whatever their fine specificity was, their presence (but not their titre) similarly constituted a marker of faster joint destruction.

Key words: rheumatoid arthritis, citrullinated, fibrinogen, autoantibodies, ACPA, anti-CCP, fine specificity, prognostic value, joint destruction, SHS.

Rheumatology key messages

- In RA, anti-citrullinated fibrin autoantibody subfamilies similarly associate with shared epitope alleles without additional effect of smoking.
- Whatever their fine specificity, anti-citrullinated fibrin autoantibody subfamilies do not predict disease activity in RA.
- Anti-citrullinated fibrin autoantibody subfamilies all predict faster joint erosion in RA, whatever their fine specificity.

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Introduction

The prognosis of RA has recently greatly improved thanks to numerous new therapeutic strategies. However, biomarkers able to predict the disease progression in early RA for optimal choice among the available therapies have still to be identified.

Several factors such as female gender (sex ratio of 3:1), some HLA haplotypes (HLA-DRB1) and cigarette smoking are understood to predispose for RA, but its pathophysiology still remains unclear [1]. Two families of autoantibodies are of first importance in the disease pathophysiology: RFs and ACPA [2]. RFs are autoantibodies directed to the Fc portion of IgG. IgM isotype RF (RF-IgM) is the most commonly used in clinical practice, but RF-IgA is also often assayed [3]. RF-IgM is present in 70–80% of RA sera, but shows a weak diagnostic specificity (80–90%) [3, 4]. In contrast, ACPAs are IgG directed to epitopes generated on the targeted antigens by the post-translational deimination (citrullination) of arginyl residues [5, 6] which are also found in 70–80% of RA sera, but are the most specific serological marker of RA, with a diagnostic specificity of 95–98% [4]. ACPAs appear early in the disease course [7], and their presence as well as a rating of their titre, were recently included in the ACR/EULAR 2010 classification criteria, beside those of RF-IgM [8]. In addition, ACPAs were shown to predict progression of undifferentiated arthritis (UA) to RA and were shown to be associated with more severe diseases [9, 10]. However, given the heterogeneity of the disease's clinical features, more reliable prognostic markers able to predict the more severe outcomes are still missing.

ACPA are commonly detected with the anti-CCP2 immunoassays developed with undisclosed CCPs. However, several proteins have been identified as *in vivo* targets of ACPAs [11–13]. Particularly, we demonstrated that citrullinated fibrin is the major target of ACPAs in RA synovial tissue and that ACPAs can be detected by ELISA on *in vitro* citrullinated human fibrinogen [14, 15]. Those autoantibodies to human citrullinated fibrinogen (AhFibA) preferentially target epitopes borne by the two citrullinated peptides α 36–50Cit and β 60–74Cit, belonging to the α - and β -chains of fibrin, respectively [16]. In addition, anti- α 36–50Cit and anti- β 60–74Cit autoantibodies were demonstrated to constitute two non-crossreactive subfamilies of AhFibA that account for the reactivity of >90% of AhFibA-positive sera [17, 18]. Thus, reactivity of sera toward the two peptides allows definition of subgroups among RA patients that might have different disease outcomes: α -sera containing autoantibodies directed to α 36–50Cit only, β -sera containing autoantibodies toward β 60–74Cit and $\alpha\beta$ -sera containing both subfamilies of autoantibodies [17, 18].

The relationships of the AhFibA subfamilies to RA risk factors and to disease progression have never been explored. Using the French ESPOIR (Etude et Suivi des Polyarthrites Indifférenciées Récentes) cohort of very early arthritides, we analysed whether AhFibA and autoantibodies directed to α 36–50Cit or β 60–74Cit differentially associate with the HLA-DRB1 alleles and with cigarette

smoking, and whether these autoantibody subfamilies have different prognostic values.

Patients and methods

Patients

Patients were recruited in the previously described French multicentre, longitudinal, prospective ESPOIR (study and follow up of recent-onset undifferentiated polyarthritis) cohort [19]. Patients with an early arthritis of <6 months' duration ($n = 813$) and a clinical diagnosis of either RA or of UA that might progress to RA, and no treatment by DMARDs or steroids, were included at baseline [19]. Unless precised, patients were considered to have RA when they fulfilled the ACR/EULAR 2010 criteria within 3 years of follow-up [8, 20]. Local institutional review boards approved the ESPOIR cohort study, and written informed consent was obtained from all subjects before inclusion [19]. This study did not require separate approval.

Clinical, biological, radiological and genetic data

Patients were classified as ever or never smokers according to their reported smoking habits at baseline. They were also classified according to the presence of the HLA-DRB1 shared epitope alleles (SE alleles) associated with RA, encoding RAA amino acids (aa's) at positions 72–74 [21].

The classification proposed by du Montcel *et al.* [22], based on the aa's at positions 70 and 71, was also used (S1 for ARAA and ERAA, S2 for KRAA, S3P for QRRAA, S3D for DRRAA, X for all non-RAA motifs). S2 and S3P are considered predisposing alleles, and S1, S3D and X as protective alleles [23, 24].

Disease disability and activity were evaluated at baseline and after 2 years of follow-up using the HAQ [25] and the DAS28 [26], respectively. Radiological damages were assessed by a single experienced rheumatologist unaware of clinical and genetic data, according to the modified Sharp/van der Heijde score (SHS) [27], at baseline and after 2 and 3 years of follow-up. The results were expressed as total Sharp score. The smallest detectable change was 1 point [28].

Autoantibody assays

IgG ACPAs were quantified by ELISA, at baseline. AhFibA and their subfamilies directed to the fibrin-derived citrullyl (Cit)-containing peptides, α 36–50Cit (aa's 36–50 of the α -chain of fibrin) and β 60–74Cit (aa's 60–74 of the β -chain) were detected according to previously described protocol [14, 16, 18]. Anti-CCP2 were assayed with the Immunoscans CCPlus ELISA (Eurodiagnostica, Arnhem, The Netherlands). Thresholds allowing 95% diagnostic specificity, previously determined using 436 control sera from patients with non-RA rheumatic diseases, were considered for all the ACPA assays [18]. RF-IgM and RF-IgA assays were performed with the commercial Quanta Lite RF-IgM and RF-IgA ELISAs (Inova, San Diego, USA).

Statistical analysis

The diagnostic values of the tests were compared using the McNemar chi-square test. Positive predictive values and negative predictive values were evaluated for patients having an UA at baseline and developing RA or not, after 3 years. Correlations were analysed by Spearman rank tests (concerning autoantibodies, only positive patients were considered), and median differences compared by Kruskal–Wallis and Mann–Whitney's U tests. P-values adjustment for multiple comparisons was done by the Holm correction method [29]. Odds ratio (OR) is the relative proportion of patients within a studied group having a high score of a dichotomized variable, compared with the negative group. For clinical and radiological data, a score is considered high or low compared with the median of all RA patients; autoantibody titres are considered positive at the 95% specificity thresholds. The level of concordance of two variables was calculated as the proportion of patients classified high or positive for both variables together with those classified low or negative for both variables, among all patients. $P < 0.05$ was considered significant. Statistical analyses were performed with Statistica for Windows (StatSoft, USA).

Results

AhFibA, anti- α 36-50Cit and anti- β 60-74Cit autoantibodies' diagnostic performance for RA at the earliest stages of the disease

The diagnostic sensitivity of autoantibody detection in the baseline sera was compared in the group of patients diagnosed as having RA at baseline and in that of patients diagnosed after 3 years of follow-up (Table 1), according to the ACR 1987 criteria [20] and to the ACR/EULAR 2010 criteria [8].

At baseline, 579 patients were diagnosed as having RA according to the ACR 1987 criteria: 52% were

AhFibA-positive, and 49% were anti-CCP2-positive. Regarding AhFibA subfamilies, 32% had anti- α 36-50Cit autoantibodies, and 39% had anti- β 60-74Cit autoantibodies (Table 1). Even though more patients were classified as having RA with the ACR/EULAR 2010 criteria either at baseline ($n = 645$) or after 3 years of follow-up ($n = 701$), the diagnostic sensitivities of each family or subfamily of autoantibodies did not differ. Compared with the diagnostic sensitivities obtained with established diseases in a previous work [18], the diagnostic sensitivities of anti- α 36-50Cit- and anti- β 60-74Cit autoantibodies appeared significantly lower in this cohort of early arthritis patients (32 vs 51% and 39 vs 71% for anti- α 36-50Cit- and anti- β 60-74Cit autoantibodies, respectively), as previously observed for AhFibA and anti-CCP2 [15, 18]. However, whatever the antibody subfamily, we did not observe any significant difference in the median autoantibody titres of positive sera between established and very early RA patients (supplementary Fig. S1, available at *Rheumatology* Online).

To analyse whether the presence of one particular subfamily of ACPsA could distinguish patients that might progress from UA to RA, we considered the ACR 1987 criteria because ACPA results are included in the ACR/EULAR 2010 criteria. Among the 188 patients with UA at baseline, 87 fulfilled the ACR 1987 criteria for RA after 3 years. Positive predictive values of anti- α 36-50Cit and of anti- β 60-74Cit autoantibodies were 82 and 79%, respectively, while those of AhFibA and anti-CCP2 were only 72 and 73%, respectively (Table 1). These differences were not significant. In the subsequent analyses, patients were considered as having RA when they fulfilled the ACR/EULAR 2010 criteria at least once during the 3-year follow-up ($n = 701$).

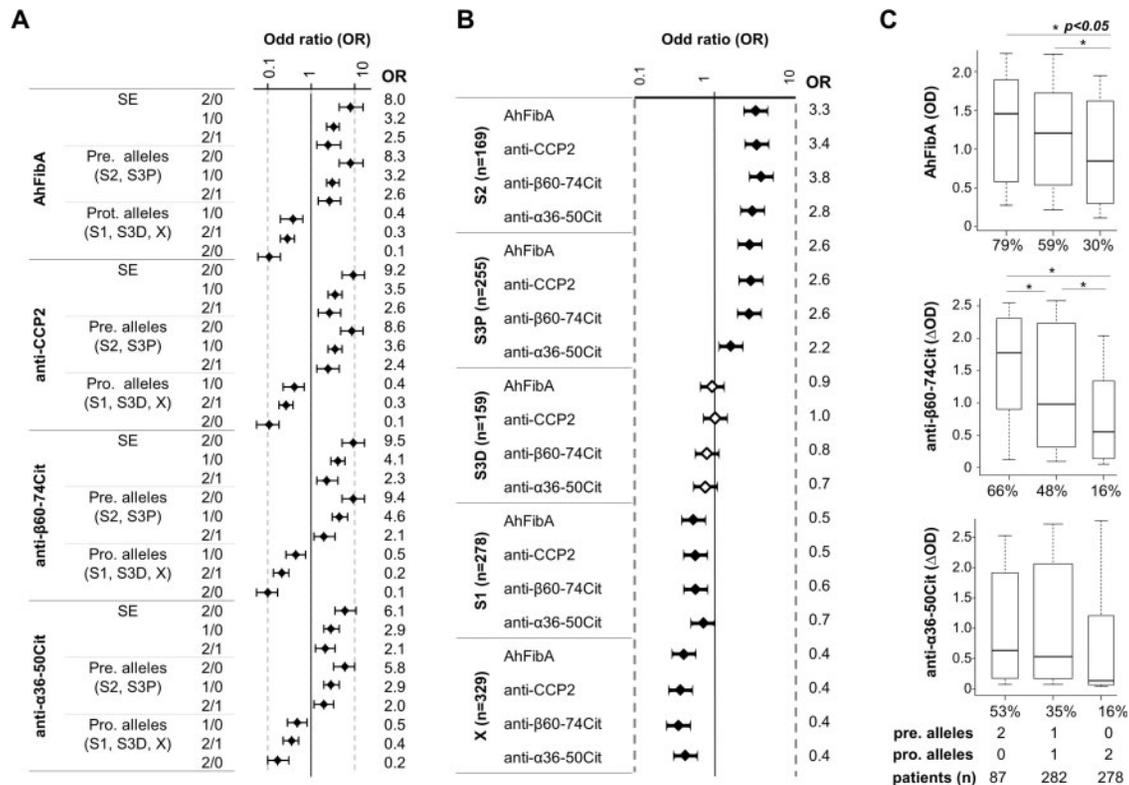
Association of AhFibA subfamilies with HLA-DRB1 SE alleles and cigarette smoking

Fig. 1A shows the relative proportions of AhFibA-, anti-CCP2-, anti- α 36-50Cit- and anti- β 60-74Cit-positive

TABLE 1 ACPA subfamily diagnostic values according to the applied diagnostic criteria and the disease time course

ACPs serological status	ACR/EULAR 2010 criteria		ACR 1987 criteria			
	Baseline $n = 645$	After 3 years $n = 701$	Baseline $n = 579$	After 3 years $n = 666$		
	Se (95% CI)	Se (95% CI)		Se (95% CI)	Se (95% CI)	PPV (95% CI)
AhFibA+	53 (49, 57)	50 (46, 54)	52 (48, 56)	50 (46, 54)	72 (56, 83)	52 (39, 58)
Anti-CCP2+	50 (46, 54)	46 (42, 50)	49 (44, 53)	46 (42, 50)	73 (56, 86)	50 (40, 59)
Anti- α 36-50Cit+	31 (28, 35)	29 (26, 32)	32 (28, 36)	30 (27, 34)	82 (59, 94)	49 (42, 59)
Anti- β 60-74Cit+	39 (36, 43)	37 (33, 40)	39 (35, 43)	37 (33, 41)	79 (60, 91)	50 (41, 59)
α -sera	8 (6, 11)	8 (6, 10)	9 (7, 11)	9 (7, 11)	78 (40, 96)	46 (75, 62)
β -sera	16 (14, 20)	15 (13, 18)	16 (13, 19)	15 (13, 18)	75 (47, 92)	47 (44, 61)
$\alpha\beta$ -sera	23 (20, 27)	21 (18, 25)	23 (20, 27)	22 (19, 25)	85 (54, 97)	48 (44, 61)

AhFibA+, anti-CCP2+, anti- α 36-50Cit+ and anti- β 60-74Cit+: AhFibA-, anti-CCP2-, anti- α 36-50Cit- and anti- β 60-74Cit-positive sera, respectively. α -sera: sera containing anti- α 36-50Cit but no anti- β 60-74Cit autoantibodies. β -sera: sera containing anti- β 60-74Cit but no anti- α 36-50Cit autoantibodies. $\alpha\beta$ -sera: sera containing both subfamilies of AhFibA. In paired comparisons, all diagnostic sensitivities significantly differ from each other ($P < 10^{-4}$) except those of β -sera vs $\alpha\beta$ -sera. PPV and NPV were evaluated using the 158 patients with UA at baseline; none of them significantly differ from the others. Se: sensitivity; PPV: predictive positive values; NPV: negative predictive values.

Fig. 1 Distribution of AhFibA subfamilies at baseline, according to HLA-DRB1 alleles

Proportion of ACPA-positive patients among those (A) having one or two copies of SE-containing alleles, predisposing (pre. alleles) or protective (pro. alleles) alleles compared with one or none (2/0: two copies vs none for example) or (B) having the S2, S3P, S1, S3D or X alleles. Black squares depict significant OR. (C) Titres of AhFibA subfamilies in positive patients (% indicated under each graph) according to the number of pro. and pre. alleles. Boxes depict the 25, 50 and 75th percentiles, whiskers the 10 and 90th, widths the number of patients. SE: shared epitope; AhFibA: autoantibodies to human citrullinated fibrinogen. OD: optical density; delta OD: difference of OD obtained on citrullinated and non-citrullinated peptides.

patients according to HLA-DRB1 alleles. Concerning HLA-DRB1 SE-containing alleles (SE alleles), 303, 282 and 81 patients had 0, 1 and 2 SE alleles, respectively. The numbers of patients were 292, 287 and 87 for 0, 1 and 2 predisposing alleles and 92, 296 and 278 for 0, 1 and 2 protective alleles, respectively. As depicted by the figure, all ACPA subfamilies were positively associated with the presence of SE or predisposing alleles ($OR > 1$) and negatively associated with the presence of protecting alleles ($OR < 1$). The strength of the association depended on the number of SE copies: for example for AhFibA positivity, the OR was 3.2 when one SE copy was present (compared with 0 copy) and 8 for two copies (compared with 0). However, whatever their fine specificity, all ACPA subfamilies exhibited similar ORs, for example: 8.0, 9.2, 6.1 and 9.5 for AhFibA, anti-CCP2, anti-α36-50Cit and anti-β60-74Cit antibodies, respectively, when two SE copies were present compared with zero.

Using the classification of du Montcel *et al.* [22], we then analysed the relative proportions of AhFibA-, anti-CCP2-, anti-α36-50Cit- and anti-β60-74Cit-positive patients according to the presence of S2, S3P, S1, S3D and X alleles (Fig. 1B). A clear association between ACPA positivity and

the predisposing alleles S2 and S3P was observed ($OR > 1$), whereas negative ORs were obtained with the protecting alleles S1 and X. However, all ACPA subfamilies were similarly associated with the different alleles (Figs 1B and 2B).

Concerning the titres of autoantibodies in positive patients, AhFibA, anti-α36-50Cit and anti-β60-74Cit autoantibody titres increased when the number of predisposing alleles increased (Fig. 1C), although the association only reached significance for AhFibA and anti-β60-74Cit autoantibodies (median titre of 0.887 and 0.551, respectively, for two protecting alleles vs 1.454 and 1.775 for two predisposing alleles).

Contrarily to SE alleles presence, cigarette smoking habit alone at baseline did not significantly increase the percentage of patients positive for AhFibA, anti-β60-74Cit or anti-α36-50Cit autoantibodies (OR of 1.21, 1.43 and 1.19, respectively, Table 2). In addition, although the combination of SE alleles with smoking seemed to have a cumulative effect when patients positive for both risk factors were compared with patients negative for both risk factors (OR of 2.45, 4.00 and 3.20), the risk appeared to be mainly due to the presence of SE alleles when we compared the

TABLE 2 Influence of HLA-DRB1 SE alleles presence and cigarette smoking on positivity for AhFibA, anti- α 36-50Cit and anti- β 60-74Cit autoantibodies

Autoantibody	Smoking	SE		Smoking	SE	OR (95% CI)	P-values
AhFibA	+	-	vs	-	-	1.21 (0.77, 1.92)	0.406
	-	+	vs	-	-	2.05* (1.37, 3.09)	<0.001
	+	+	vs	-	-	2.45* (1.62, 3.72)	<10 ⁻⁴
	+	+	vs	-	+	1.19 (0.86, 1.66)	0.296
anti- β 60-74Cit	+	-	vs	-	-	1.43 (0.80, 2.57)	0.225
	-	+	vs	-	-	3.36* (2.02, 5.59)	<10 ⁻⁴
	+	+	vs	-	-	4.00* (2.39, 6.69)	<10 ⁻⁴
	+	+	vs	-	+	1.19 (0.84, 1.69)	0.333
anti- α 36-50Cit	+	-	vs	-	-	1.19 (0.65, 2.18)	0.579
	-	+	vs	-	-	2.35* (1.39, 3.96)	0.001
	+	+	vs	-	-	3.20* (1.89, 5.40)	<10 ⁻⁴
	+	+	vs	-	+	1.36 (0.93, 2.00)	0.116

*P < 0.05 were considered significant. SE: patients with at least one SE allele. OR: Odds Ratio. Groups were constituted according to cigarette smoking (+) or not (-) and SE presence (+) or absence (-). For smoking +/SE +, n = 161; for smoking +/SE -, n = 154; for smoking -/SE +, n = 202; for smoking -/SE -, n = 149.

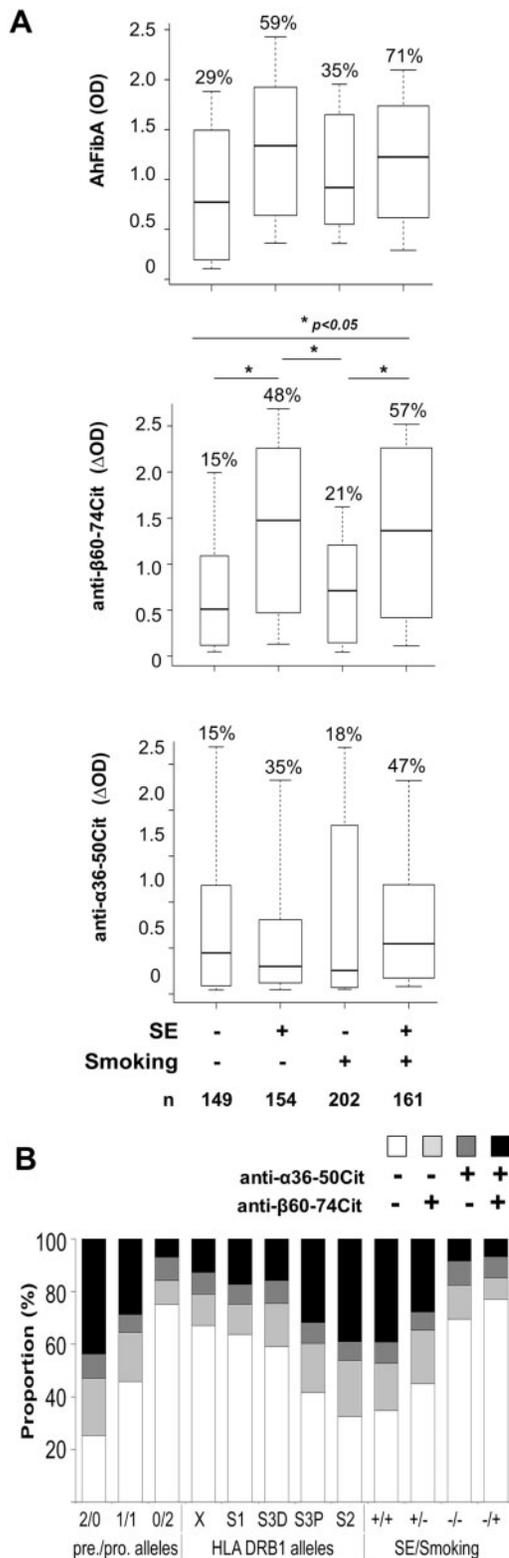
group of patients positive for both risk factors with patients positive for SE alleles presence only (OR of 1.19, 1.19 and 1.36, respectively). In all cases, the combination of SE alleles with smoking similarly impacted the presence of AhFibA and that of their subfamilies. Although the effect seemed more pronounced for both subfamilies, the 95% CIs were overlapping, and this trend needs to be confirmed with other cohorts. We thus investigated whether risk factors would also similarly impact autoantibody titres (Fig. 2A). The titres of AhFibA and those of their subfamilies among positive patients were evaluated according to the presence of SE alleles and/or cigarette smoking habit at inclusion. Cigarette smoking was not associated by itself with a significant increase in the median titre of any of the AhFibA subfamilies. To the contrary, the presence of at least one SE allele was associated with an increase in AhFibA titre and of those of their subfamilies, although the increase only reached significance for anti- β 60-74Cit autoantibody (Fig. 2A, middle panel). We then wondered whether the proportion of sera containing anti- α 36-50Cit and/or anti- β 60-74Cit autoantibodies changed according to the number of SE copies, to the HLA-DRB1 sequence and to smoking habit. As seen in the Fig. 2B, although the proportion of ACPA-positive sera was highly influenced by the presence of predisposing alleles, the relative proportion of each AhFibA subfamily was not.

Faster bone erosion is associated with the presence of AhFibA and of their subfamilies regardless of their titres, but functional impairment and disease activity are not

To determine the potentially different prognostic values of ACPA subfamilies, we analysed the outcome of the disease in groups of patients defined by their ACPA profiles at baseline (Table 3). All groups of ACPA-positive patients as well as ACPA-negative patients were highly homogeneous for sex ratio, cigarette smoking and for the baseline levels of HAQ, DAS28 and SHS. All ACPA-positive groups were also highly homogeneous for age even though their median ages were slightly lower than that of ACPA-negative patients. As expected, the proportion of RF-positive sera (RF-IgM and RF-IgA) and that of patients carrying SE alleles was significantly higher in ACPA-positive compared with ACPA-negative patients (Table 3). Thus, the groups were suitable for comparison during the follow-up.

The DAS28, HAQ and SHS distribution in each group of patients are compared in Fig. 3 at inclusion and after two (DAS28 and HAQ) or three (SHS) years of follow-up. In the right part of each panel, ORs reflect the relative proportions of patients of a group having a high DAS28 (or HAQ or SHS) compared with the patients who do not belong to the same group. At baseline, all patient groups exhibited

Fig. 2 Distribution of AhFibA subfamilies at baseline according to the combination of HLA-DRB1 and smoking status



(A) Titres of AhFibA subfamilies in positive patients (% indicated on each graph) according to the presence (+) or

similar HAQ, DAS and SHS levels. After 2 years of follow-up, DAS28 and HAQ levels were still similar for all groups, whereas after 3 years of follow up, SHS was significantly higher for all ACPA-positive groups compared with ACPA-negative patients. To analyse whether some groups had a more severe worsening, we calculated for each patient the individual variation in each parameter from baseline to 2 or 3 years of follow up (variation of 0–2 or 3 years). All groups had similar variation in DAS28 and HAQ during the first 2 years, whereas ACPA-positive patients had faster bone erosion (variation of SHS) irrespective of the fine specificity of ACPA. For example, the 25th, 50th and 75th percentiles of the variation in SHS from baseline to after 3 years of follow-up were 1, 4 and 8 for AhFibA-negative patients and 3, 8 and 16 for AhFibA-positive ones. They were 3.5, 8 and 16 for anti-β60-74-positive patients and 3, 8 and 15 for anti-α36-50-positive patients. Thus, patients having anti-β60-74Cit and/or anti-α36-50Cit or AhFibA or anti-CCP2 antibodies, showed bone erosion about two times faster than that of AhFibA- or anti-CCP2-negative patients.

No significant correlation appeared between the autoantibody titres and HAQ, DAS28 and SHS (left/lower part of the heatmap in supplementary Fig. S2, available at *Rheumatology* Online). In contrast, RF-IgM and IgA-RF titres on one hand, and AhFibA and anti-CCP2 antibody titres on the other hand, appeared highly significantly correlated ($r=0.518$; $P < 10^{-20}$ and $r=0.597$; $P < 10^{-28}$, respectively, supplementary Fig. S3, available at *Rheumatology* Online). In addition, anti-β60-74Cit autoantibody titres were highly significantly correlated with those of AhFibA ($r=0.535$; $P < 10^{-16}$) and anti-CCP2 ($r=0.446$; $P < 10^{-9}$). RF and ACPA titres were not correlated and were independent of the clinical and radiological parameters. However, as is usually observed, the two autoantibody families were most often present together in RA sera [30]. Indeed, the presence or the absence of AhFibA and that of RF-IgM was concordant in 82% of RA sera (right/upper part of the heatmap).

DAS28 and HAQ were highly correlated with each other at baseline ($r=0.571$; $P < 10^{-58}$, supplementary Fig. S2, available at *Rheumatology* Online) and during follow-up (supplementary Fig. S2, available at *Rheumatology* Online). Variation in DAS28 and variation in HAQ were also correlated and highly concordant. For example, 79% of the patients that had the highest or lowest variation in DAS28 also had the highest or lowest variation in

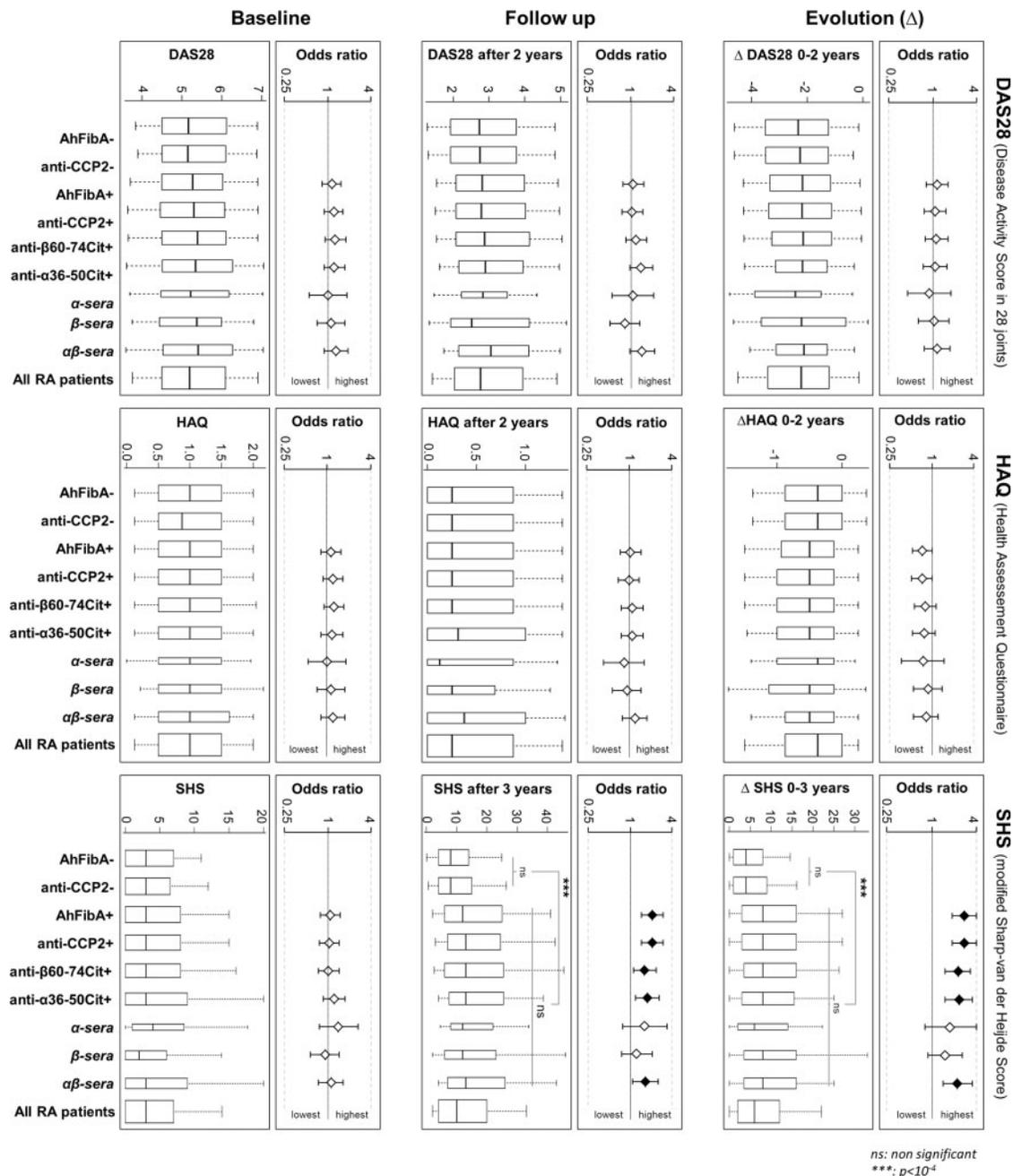
not (–) of SE alleles (SE) and that of smoking habit (Smoking). Boxes depict the 25, 50 and 75th percentiles, whiskers the 10 and 90th, widths the number of patients. **(B)** Proportion of sera containing AhFibA subfamilies according to the presence of predisposing (pre. alleles), protective (pro.alleles), S2, S3P, S1, S3D or X alleles and to the combination of SE and smoking status. SE: shared epitope; AhFibA: autoantibodies to human citrullinated fibrinogen; OD: optical density; ΔOD: difference of OD obtained on citrullinated and non-citrullinated peptides.

TABLE 3 Baseline characteristics of the 701 patients diagnosed as having RA according to the ACR/EULAR 2010 after 3 years' follow-up, classified according to their ACPA profile

Patient characteristics	All RA n = 701			ACPA-			ACPA+			P-values
	Anti-CCP2- n = 377	AhFibA- n = 352	Anti-CCP2+ n = 324	AhFibA+ n = 349	Anti-β60-74Cit+ n = 257	Anti-α36-50Cit+ n = 203	α-sera n = 54	β-sera n = 108	αβ-sera n = 149	
Proportion of woman, %	81	81	74	74	74	73	81	79	70	NS
Age, median (IQR), years	53 (41-59)	53 (43-60)	48 (39-56)	48 (38-56)	48 (38-56)	48 (38-55)	49 (42-55)	48 (40-57)	48 (38-55)	*
Cigarette smoking, %	46	46	50	50	51	52	50	47	53	NS
Cigarette smoking, median (IQR), packs per year	12 (5-25)	12 (5-26)	15 (6-30)	15 (5-30)	17 (7-30)	15 (6-30)	10 (3-22)	15 (6-30)	19 (9-30)	NS
HLA-DRB1 SE+, %	37	39	69	71	74	72	50	65	80	*
IgM-RF positive sera, %	23	22	91	87	88	88	76	81	92	*
IgM-RF titre, AU, median of positive (IQR)	24 (13-57)	23 (13-52)	74 (33-266)	74 (32-266)	74 (30-265)	74 (32-253)	62 (30-177)	71 (27-233)	79 (34-266)	*
IgA-RF positive sera, %	24	23	89	85	86	85	72	81	90	*
IgA-RF titre, AU, median of positive (IQR)	16 (10-28)	15 (10-28)	35 (20-69)	34 (19-68)	34 (20-63)	34 (20-79)	26 (16-64)	29 (19-47)	38 (20-80)	*
DAS28, median (IQR)	5.2 (4.5-6.1)	5.1 (4.5-6.1)	5.3 (4.5-6.1)	5.2 (4.5-6.0)	5.4 (4.5-6.1)	5.3 (4.5-6.3)	5.2 (4.5-6.1)	5.4 (4.4-6.0)	5.4 (4.5-6.3)	NS
HAQ level, median (IQR)	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-1.5)	1 (0.5-1.6)	NS
SHS, median (IQR)	3 (0-7)	3 (0-7)	3 (0-8)	3 (0-8)	3 (0-8)	3 (0-9)	4 (1-8)	2 (0-6)	3 (0-9)	NS

*P < 0.05 (at least two groups differ). ACPA-, anti-CCP2- and AhFibA-; ACPA+, anti-CCP2+, AhFibA+, anti-α36-50Cit+ and anti-β60-74Cit+; ACPA-, Anti-CCP2-, AhFibA-, anti-β60-74Cit+ positive sera. α-sera: sera containing anti-α36-50Cit but no anti-β60-74Cit autoantibodies; β-sera: sera containing anti-β60-74Cit but no anti-α36-50Cit autoantibodies; αβ-sera: sera containing both subfamilies of AhFibA. AU: arbitrary unit. HLA-DRB1 SE+: patients with at least one SE allele; NS: not significant.

Fig. 3 Prognostic value of AhFibA and their subfamilies measured at baseline



DAS28, HAQ and SHS were evaluated at baseline and after follow-up (2 or 3 years). The Evolution column corresponds to their variations (Δ) from baseline. AhFibA+, anti-CCP2+, anti- α 36-50Cit+, anti- β 60-74Cit+, AhFibA– and anti-CCP2– correspond to patients having (+) or not (–) the mentioned antibodies. α -sera corresponds to patients having anti- α 36-50Cit but no anti- β 60-74Cit autoantibodies; β -sera, patients having anti- β 60-74Cit but no anti- α 36-50Cit autoantibodies; $\alpha\beta$ -sera, patients having both subfamilies of AhFibA. Boxes depict the 25, 50 and 75th percentiles, whiskers the 10 and 90th, widths the number of patients. AhFibA: autoantibodies to human citrullinated fibrinogen.

HAQ, after 2 years of follow-up. However, both DAS28 and HAQ were independent of SHS and of all the serological parameters (supplementary Fig. S2, available at *Rheumatology* Online).

In contrast, ACPAs were associated with the most erosive diseases (highest variation of SHS after 3 years of follow-up). Indeed, the presence or absence of ACPAs (AhFibA, anti-CCP2, anti- β 60-74Cit or anti- α 36-50Cit)

but also that of RF (RF-IgM or RF-IgA) and the speed of erosion (fast or slow) were concordant in ~60% of RA sera.

Discussion

Using the ESPOIR cohort composed of very early arthritides, we investigated the relationship of ACPA subfamilies with genetic, clinical and radiological data. More particularly, we investigated whether the two non-cross-reactive subfamilies of AhFibA (anti- α 36-50Cit and anti- β 60-74Cit autoantibodies) that summarize almost all the ACPA positivity and allow defining of subgroups of patients among ACPA-positive patients are differently associated with clinical data and differently predict the disease progression.

In this cohort of Caucasian patients with early RA, AhFibA and anti-CCP2 had similar diagnostic performances and a high level of concordance (93%). AhFibA, anti-CCP2, anti- α 36-50Cit and anti- β 60-74Cit autoantibodies were detected in proportions of RA sera (50, 46, 29 and 37%, respectively) significantly lower than those previously described in Caucasian (83, 74, 51 and 71%, respectively) as well as in Black African (73, 82, 45 and 73%, respectively) patients with established RA [18, 31]. However, proportions of each AhFibA subfamily among AhFibA-positive sera were similar in the three cohorts: 55% and 70% for anti- α 36-50Cit and anti- β 60-74Cit autoantibodies, respectively, in the present cohort of Caucasians with very early RA; 59 and 81%, respectively, for Caucasian and 56 and 86%, respectively, for Black African patients with established RA. When considered together, anti- α 36-50Cit- and anti- β 60-74Cit-positive patients similarly accounted in the three cohorts for almost all AhFibA-positive patients (83, 90 and 93%, respectively). The immunodominance of the epitope borne by β 60-74Cit was confirmed in early RA because 74 and 71% of anti-CCP2 and AhFibA-positive RA sera, respectively, were anti- β 60-74Cit-positive. Thus, ACPA features are very stable, irrespective of the duration of the disease and of the genetic factors.

SE alleles appeared to be a risk factor for the presence of AhFibA, as has been observed for anti-CCP in cohorts of various origins (Caucasians, Black Africans, Native Americans) [23, 31, 32]. Similarly, anti- α 36-50Cit and anti- β 60-74Cit autoantibodies were also associated with the presence of SE alleles. However, no preferential association with any one of the AhFibA subfamilies was observed. When considering the du Montcel's classification for HLA-DRB1 alleles, AhFibA presence (and similarly that of each AhFibA subfamily) was associated with S2 and S3P but not with S3D, S1 and X presence, confirming the impact of protective epitopes on the production of AhFibA, as previously observed for anti-vimentin or anti-CCP antibodies [23, 33]. Finally, not only the presence, but also the titres of AhFibA and their subfamilies were higher when the number of copies of SE alleles was greater.

These results nourish the question of the mechanisms underlying the genetic control of ACPA production.

Indeed, several studies have addressed the question of a role for SEs in the presentation of citrullinated antigens [34]. However, although an increased affinity for SEs of the citrullinated form of a vimentin peptide compared with its native counterpart has been described, this was not observed for fibrinogen peptides. In addition, no difference in T cell proliferation has been observed in response to native or citrullinated fibrinogen peptides, suggesting that the presentation of citrullinated epitopes is not the only molecular mechanism underlying the SE effect on ACPA production. Moreover, several gene polymorphisms have been associated with RA. Thus, besides SE alleles, other yet to be determined genes within the genetic background might be involved in the regulation of ACPA production; however, the way they might be involved in the production of different ACPA specificities remains to be elucidated. Environmental factors (cigarette smoking in particular) are also considered risk factors for ACPA positivity. However, cigarette smoking was not consistently found to synergize with the presence of SE alleles [35, 36]. In our cohort of early RA patients, contrary to the way SE alleles were significantly associated with both the presence and the titres of AhFibA and those of their subfamilies, smoking by itself did not appear to have any significant effect. The link between SE alleles/smoking and titres of ACPA subfamilies has never been explored with the exclusion of ACPA negative patients; differences in the proportions of positive patients may introduce a bias. Other studies analysed the association of SE alleles, cigarette smoking and ACPA fine specificity and, in agreement with our results, found that several ACPA subfamilies (directed to peptides from vimentin and α -enolase) were associated with SE alleles but not with smoking by itself. In addition, although a significant interaction between cigarette smoking and SE alleles was found in the presence of ACPA, this interaction was not present after stratification for ACPA status, indicating that cigarette smoking and SE alleles did not influence autoimmunity to specific citrullinated antigens [37-39].

Whatever the test used for their detection, ACPA presence in the ESPOIR cohort was not related to DAS28 or HAQ, and no difference was observed between AhFibA subfamilies. Data published concerning the relationship of ACPA with disease activity are heterogeneous [9, 40-44]. However, DAS28 is a punctual measurement and can change rapidly over time; moreover, both DAS28 and HAQ are influenced by treatments. Therefore, the cohort characteristics strongly influence analysis, which could explain the discrepancies.

All the four ACPA families and subfamilies were predictive of faster bone erosion: the speed of erosion was about two times higher than in ACPA-negative patients. Notably, the results were similar for the two AhFibA subfamilies. These results are concordant with those of Scherer *et al.* [45], who did not find any difference in bone erosion degree related to the presence of ACPA to citrullinated peptides from vimentin or α -enolase. They are also concordant with those of van Beers *et al.* [46], who identified 12 distinct ACPA profiles among RA patients using

microarray sensor chips with 20 citrullinated peptides, but did not find any statistically significant association of clinical features at diagnosis or during the disease course with the five most frequent profiles. However, the autoantibodies studied in both cases were directed towards different protein antigens and might be, at least partially, cross-reactive and overlapping. Anti- α 36-50Cit and anti- β 60-74Cit autoantibodies (studied in our research) are two non-crossreactive subfamilies of autoantibodies directed towards the same autoantigenic target. It is, therefore, puzzling that some patients produce either one and/or the other of those autoantibodies. We investigated whether RA clinical features or different genetic factors could be differentially associated with those profiles. However, our present results and those we obtained in a previous work in a black African cohort [31] do not support these hypotheses.

If the link between the presence of ACPA or RF and bone erosion has been clearly established [9, 42-44, 47, 48], the impact of the autoantibody titres is less clear [10, 49]. Recently, Humphreys *et al.* [50] demonstrated that although seropositivity for both RF and ACPA was associated with excess mortality, antibody level was not. In our study, neither the titres of AhFibA, nor those of anti- α 36-50Cit autoantibodies, anti- β 60-74Cit autoantibodies or RF, correlated with bone erosion at baseline or during the follow-up, or with the speed of erosion. In addition, we did not find any cumulative effect of the presence of both antibodies in the same sera. However, since almost all (82% of) RF-positive sera were ACPA-positive, it was not possible to constitute single positive groups of sufficient size to decipher the impact of each antibody independently.

To conclude, AhFibA, anti- α 36-50 Cit and anti- β 60-74Cit autoantibodies, like other ACPAs, have a lower diagnostic sensitivity in early RA compared with in established disease, but their relative proportions among positive patients are similar in the two situations. In early UA, they equally allow prediction of RA development. Moreover, they are similarly linked with HLA-DR SE alleles but are all independent of cigarette smoking. Their relationships with IgM and IgA-RF, DAS28, HAQ and SHS are similar. Linked to SHS, the presence of AhFibA, of anti- α 36-50Cit and of anti- β 60-74Cit autoantibodies (but not their titres) is predictive of faster bone erosion as well as of RF.

The very homogeneous characteristics of the AhFibA subfamily autoantibodies regarding genetic, serological and clinical associations, suggest that they might be similarly involved in the pathophysiology of the disease, possibly by being similarly able to form immune complexes with citrullinated fibrin in the synovial tissue.

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Supplementary data

Supplementary data are available at *Rheumatology Online*.

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