Impact of anti-human IgG hinge peptide antibodies on identification of patients with early seronegative rheumatoid arthritis

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Abstract

Objective

The early diagnosis of seronegative rheumatoid arthritis (SNRA), characterised by the absence of rheumatoid factor and anti-citrullinated antibody, involves a greater challenge compared to seropositive RA (SPRA). This study aimed to assess the discriminatory potential of anti-human IgG hinge antibodies (AHAs) for patients with early SNRA.

Methods

DMARDs-naive patients with SPRA (n=43), SNRA (n=21), and non-RA (n=49), with disease duration < 2 years, were included. Antigens comprised IgG1 or IgG4 F(ab')$_2$ cleaved by pepsin or MMP-3 and their hinge peptide analogues. Eight IgG anti-hinge antibodies (AHAs) against these antigens were measured in sera from the patients and 58 healthy controls (HCs) using ELISA. Serum CRP and MMP-3 levels, and clinical disease activity index (CDAI), were obtained from medical records. The area under the curve (AUC) obtained from logistic regression and receiver operating characteristic curve analyses were used as a discriminant indicator.

Results

The levels of the IgG AHAs were as follows: SPRA≥SNRA≈non-RA>HC. None of the AHAs were effective in discriminating SNRA from non-RA. However, the combination of MMP-3 and AHAs against IgG4 hinge peptide analogues demonstrated the utility (AUC=0.94). Furthermore, combination of MMP-3, AHAs against IgG1 hinge peptide analogues and CDAI maximally exerted discriminatory power (AUC=0.997).

Conclusion

Specific AHAs in combination with MMP-3 and CDAI are potentially useful to discriminate SNRA from non-RA.

Key words

rheumatoid arthritis, autoantibodies, seronegative, IgG, biomarkers, disease activity

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Introduction

The 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria have facilitated the early diagnosis and treatment of rheumatoid arthritis (RA), leading to clinical remission and improved daily activities and quality of life (1). However, these criteria incorporate RA-related autoantibodies such as rheumatoid factor (RF) and anti-CCP antibodies, leading to high scores. This indicates a dominant classification for autoantibody-positive patients with arthritis but disadvantages for autoantibody-negative patients. RA is typically classified into SPRA and SNRA according to the presence or absence of autoantibodies. SNRA seems to be different from SPRA in the genetic backgrounds, environmental factors, modes of onset, responses to treatment, and outcomes of joint involvement (2-4). Reports indicate that early inflammatory joint diseases other than SPRA are sometimes misdiagnosed as SNRA, presenting challenges in obtaining a distinct diagnosis (5, 6).

Human IgG functions not only as an antibody that reacts with antigens but also as an antigenic protein with various epitopes (7). The most widely studied antigen is the Fc region, consisting of the second and third constant homology (CH) regions, and autoantibodies against the Fc region are known as RF (8, 9). The region connecting the CH1 and CH2 of IgG is termed a hinge. Pepsin agglutinators (PA), which are antibodies to IgG F(ab')$_2$ generated by pepsin, were discovered approximately 60 years ago (10). Their epitopes are mainly located in the hinge region and are collectively referred to as anti-hinge antibodies (AHA) (11). Serum PA is known to have a high positive rate and titer in patients with RA (12), and its production in the joints of patients with RA has been confirmed (13), suggesting a relationship with the pathogenesis of RA. The amino acid sequence of the hinge region differs among IgG subclasses and various proteases such as microbial proteases and matrix metalloproteinases (MMPs) act at different sites in the hinge region, resulting in distinct hinge neoepitopes (14, 15). These hinge neoepitopes bind to the corresponding specific AHAs (16). Regarding the disease specificity of AHAs, AHAs against the human IgG4 hinge antigen-which appeared under the action of pepsin or MMP-7 show disease specificity for RA (16-18).

The lack of useful biomarkers may present difficulty in identifying early SNRA. We have demonstrated that IgG AHA against human IgG4 F(ab')$_2$ generated by pepsin serves as a biomarker for patients with RA, particularly those using biologics with long-term disease duration, when compared to healthy individuals and patients with non-RA (18). Additionally, we also demonstrated the IgG AHA against IgG4 F(ab')$_2$ generated by pepsin as a biomarker for SNRA, although healthy individuals used as controls.

As mentioned above, each AHA specifically reacts with the IgG hinge neoepitopes present in each IgG subclass that are generated by various proteases. AHAs react with the conformational epitopes appearing in IgG F(ab')$_2$ and also with the linear epitope at the C-terminus of the amino acid sequence comprising the hinge region (11). The AHA specificity seems to be primarily due to the linear epitope (11, 14). Given these circumstances, besides IgG F(ab')$_2$, hinge peptide analogues as linear epitopes were used in this study to detect AHAs.

This study aimed to investigate whether some AHAs are specific for disease-modifying anti-rheumatic drug (DMARDs)-naive early SNRA and whether the AHAs could contribute discrimination the SNRA from other inflammatory joint diseases.

Methods

Patients and healthy controls

Among the 288 consecutive first-visit patients from April 2017 to March 2019, 176 patients with joint symptoms (pain, swelling, stiffness) were included (Fig. 1). Of these, 116 were identified as early patients with disease duration of less than 2 years. Sixty-seven patients met the 2010 ACR/EULAR classification criteria for RA. Finally, patients with early and DMARD-naive RA were divided into two groups: 43 patients

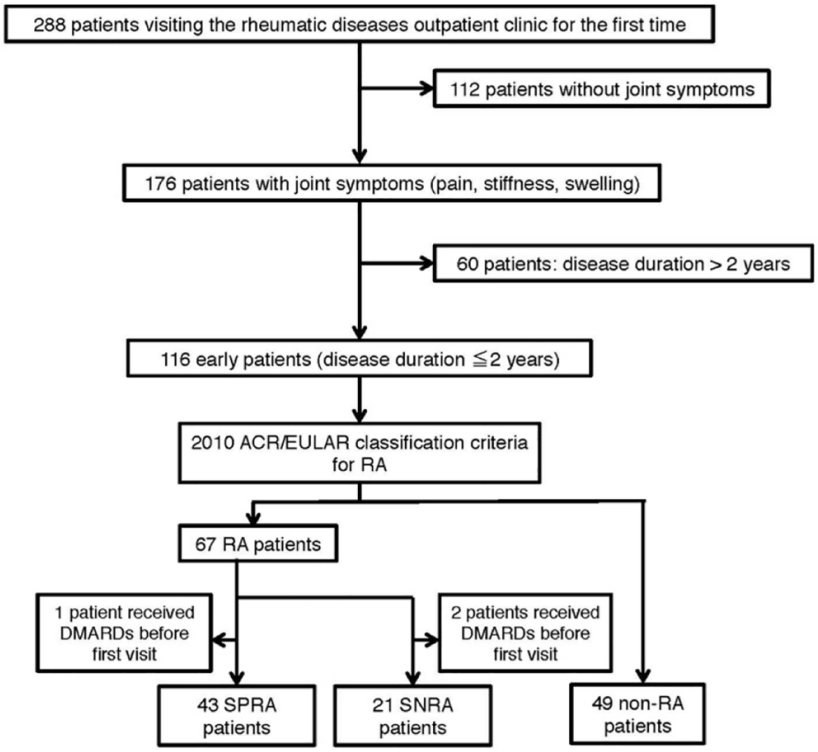

Fig. 1. Flow diagram of patient enrolment.

with SPRA with RF and/or anti-CCP2 positivity and 21 patients with SNRA without RF and anti-CCP2 positivity. Meanwhile, 49 early patients who did not meet the 2010 ACR/EULAR classification criteria were selected as patients with non-RA. The patients with non-RA were finally classified as osteoarthritis (n=14), polymyalgia rheumatica (n=8), unclassified arthritis (n=18), reactive arthritis (n=2), calcium pyrophosphate crystal deposition disease (n=2), psoriatic arthritis (n=1), pustulotic arthro-osteitis (n=1), neoplastic arthropathy (n=1), Sjögren's syndrome (n=1), and systemic lupus erythematosus (n=1). Sera from healthy controls (HCs) were obtained from 58 adults who visited the Prevention Medical Center at Iizuka Hospital for medical checkups. The HCs comprised 30 females (51.7%) and 28 males (48.3%). The median age (interquartile range) for females and males was 59.5 (53.0–65.0) and 61.5 (58.0–65.5), respectively. The age, sex, smoking, CDAI, modified health assessment questionnaire, Steibrocker's stage, serum autoantibodies (RF, anti-CCP2 antibody), serum CRP, and matrix metalloproteinase-3 (MMP-3) levels were obtained from medical records.

Synthesised peptide

Four human IgG1 and IgG4 hinge peptide analogues were synthesised by Scrum Inc. (Tokyo, Japan) in a Biotin-PEG4-hinge-14-mer peptides. The hinge peptide sequences were as follows: DKTHTCPPCPAPEL for the γ1 peptide analogue cleaved by pepsin; SCDKTHTCPPCPAP for the γ1 peptide analogue cleaved by MMP-3; KYGPPCPSCPAPEF for the γ4 peptide analogue cleaved by pepsin; and ESKYGPPCPSCPAP for the γ4 peptide analogue cleaved by MMP-3.

Measurement of IgG AHAs to IgG F(ab')$_2$ and hinge peptide analogue

IgG F(ab')$_2$ fragments derived from tocilizumab IgG1 and natalizumab IgG4 were generated through the proteolytic cleavage of pepsin or MMP-3. IgG AHAs were quantified using enzyme-linked immunosorbent assay (ELISA), as previously described (18). Each purified F(ab')$_2$ fragment was labeled with an italic subscript indicating the responsible protease for cleavage, such as IgG1 F(ab')$_{2\ pepsin}$. Eight definite AHAs were as follows: AHA1: IgG AHA against TCZ IgG1 F(ab')$_{2\ pepsin}$, AHA2: IgG AHA against TCZ IgG1 F(ab')$_{2MMP\text{-}3}$, AHA3: IgG AHA against NTZ IgG4 F(ab')$_{2\ pepsin}$, AHA4: IgG AHA against NTZ IgG4 F(ab')$_{2MMP\text{-}3}$, AHA5: IgG AHA to γ1 hinge peptide analogue cleaved by pepsin, AHA6: IgG AHA to γ1 hinge peptide analogue cleaved by MMP-3, AHA7: IgG AHA to γ4 hinge peptide analogue cleaved by pepsin, AHA8: IgG AHA to γ4 hinge peptide analogue cleaved by MMP-3.

To detect IgG antibodies against hinge peptide analogues, ELISA plates (Sumitomo Bakelite, Tokyo, Japan) were coated with streptavidin (Wako Inc., Tokyo, Japan) using the following procedure. The streptavidin was adjusted to 2 μg/mL using 10 mM phosphate buffer (pH 8.0), 100 μL was added to each well and allowed to bind overnight at 4°C. After adjusting streptavidin to 2 μg/mL using 10 mM phosphate buffer (pH 8.0), 100 μL was added to each well and allowed to bind overnight at 4°C. After washing with distilled water and 10 mM Tris buffer containing 0.9% NaCl with 0.05% Tween-20 (TBST) at pH 7.4, hinge peptide analogues were added to replicate wells at concentration 0.5 μg/mL and volume 100 μL. The mixture was incubated for 1 h at room temperature (RT: 25°C). Following washing, serum samples were diluted with TBST to a ratio of 1:200, added to the plate (100 μL/well), and incubated for 2 h at RT. After washing, 100 μL/well of alkaline phosphatase (ALP)-conjugated anti-human IgG Fc (Sigma-Aldrich) was diluted to a ratio of 1:10,000 with TBST and incubated for 1 h at RT. After washing, the AHAs were visualised with 1 mg/mL p-nitrophenyl phosphate in diethanolamine buffer at pH 9.8 for 30 min. The absorbance was measured at 405 nm using a microplate reader. AHA levels (arbitrary units/mL: AU/mL) were calculated using a calibration curve generated from pooled sera with high absorbance values of AHA against IgG F(ab')$_{2\ pepsin}$. This calibration curve was used to measure all AHA levels.

Statistical analysis

The non-parametric Kruskal-Wallis test was used to compare multiple groups. The Mann-Whitney U-test was

Table I. Baseline characteristics of early patients with rheumatoid arthritis or non-RA.

Variables	SPRA (n=43)	SNRA (n=21)	non-RA (n=49)	*P*
Age (years) at first visit, median (Q1-Q3)	66 (55-76)	69 (60-72)	63 (54-73)	NS
Female, n (%)	33 (76.7)	12 (57.1)	34 (69.4)	NS
Ever smoking/Never smoking, n (%) /n (%)	16 (38.1)/26 (61.9)	6 (28.6)/15 (71.4)	19 (40.4)/28 (59.6)	NS
Symptom duration: weeks, median (IQR)	18 (12-47)	14 (10-24)	15 (8-31)	NS
CDAI, median (IQR)	15.1 (9.1-26.0)	21.4 (14.5-29.5)	9.1 (5.3-12.8)	<0.001
mHAQ score, median (IQR)	0.63 (0.13-1.1)	0.50 (0.1-1.3)	0.31 (0-0.8)	NS
Steinbrocker's stage, n (%)				NS
I	23 (53.5)	15 (71.4)		
II	14 (32.6)	5 (23.8)		
III	6 (13.9)	1 (4.8)		
IV	0 (0)	0 (0)		
RF: HP/LP/N, n(%)	30 (69.8)/10 (23.2)/3 (7.0)	0 (0.0)/0 (0.0)/21 (100)	12 (24.5)/7 (14.3)/30 (61.2)	<0.001
Anti-CCP2 antibody: HP/LP/N, n (%)	34 (79.1)/2 (4.6)/7 (16.3)	0 (0.0)/0 (0.0)/21 (100)	2 (4.1)/0 (0.0)/ 47 (95.9)	<0.001
CRP: mg/dL, median (IQR)	0.54 (0.1-2.6)	2.55 (0.2-5.5)	0.15 (0.03-0.97)	<0.01
MMP-3: ng/mL, median (IQR)	112.3 (52.0-204.9)	220.2 (87.5-346.8)	40.1 (32.7-88.0)	<0.001

SPRA: seropositive rheumatoid arthritis; SNRA: seronegative rheumatoid arthritis; IQR: interquartile range; CDAI: clinical disease activity index; mHAQ: modified health assessment questionnaire; HP: high-positive; LP: low-positive; N: negative; RF: rheumatoid factor; CCP: cyclic citrullinated peptide; CRP: C-reactive protein; MMP-3: matrix metalloproteinase-3.

used to assess the differences between the two groups. Dichotomous variables were analysed using Fisher's exact or chi-square test. Logistic regression analyses were used to determine the variables that contribute to the identification of SNRA when non-RA is used as a control, and their diagnostic properties were examined. Receiver operating characteristic (ROC) curves were generated using the calculated probability values, and the area under the curve (AUC) was calculated for each variable or a combination of variables. Statistical significance was defined as a two-tailed *p*-value <0.05. Data were analysed on a personal computer using StatFlex version 6 (Artech, Osaka, Japan) and StatMate version 5 (ATMS, Tokyo, Japan).

Ethics

This study complied with the Declaration of Helsinki. Written informed consent was obtained from all participants and the Iizuka Hospital Ethics Committee approved this study according to the number 21017.

Table II. Association of variables with SNRA when non-RA was used as a control.

	Unadjusted			Adjusted for sex and age		
Variable	AUC	OR (95% CI)	*p*	AUC	OR (95% CI)	*p*
Age	0.58	1.02 (0.98,1.06)	0.40			
Sex	0.56	0.59 (0.20,1.69)	0.33			
Smoking	0.56	0.59 (0.19,1.79)	0.35	0.63	0.53 (0.15,1.79)	0.30
AHA1	0.62	0.99 (0.99,1.00)	0.11	0.62	0.99 (0.99,1.00)	0.14
AHA2	0.58	1.01 (0.99,1.02)	0.09	0.63	1.00 (0.99,1.02)	0.11
AHA3	0.46	1.02 (0.97,1.07)	0.43	0.61	1.01 (0.92,1.12)	0.80
AHA4	0.48	0.98 (0.93,1.04)	0.60	0.59	1.02 (0.97,1.06)	0.54
AHA5	0.55	0.98 (0.94,1.04)	0.60	0.62	0.99 (0.94,1.04)	0.73
AHA6	0.56	1.04 (0.96,1.12)	0.34	0.62	1.03 (0.96,1.11)	0.39
AHA7	0.61	1.04 (0.97,1.11)	0.24	0.62	1.04 (0.97,1.11)	0.28
AHA8	0.56	1.10 (0.97,1.24)	0.12	0.64	1.10 (0.97,1.25)	0.14
CRP	0.72	1.49 (1.15,1.93)	0.002	0.77	1.47 (1.13,1.92)	0.004
MMP-3	0.81	1.01 (1.00,1.02)	0.005	0.84	1.02 (1.00,1.03)	0.004
CDAI	0.81	1.14 (1.06,1.22)	< 0.001	0.81	1.13 (1.06,1.22)	< 0.001

AUC: area under the curve; OR: odds ratio; 95% CI: 95% confidential interval; CRP: C-reactive protein; MMP-3: matrix metalloproteinase-3; CDAI: clinical disease activity index; AHA1: IgG AHA to TCZ IgG1 F(ab')$_{2pepsin}$; AHA2: IgG AHA to TCZ IgG1 F(ab')$_{2MMP\text{-}3}$; AHA3: IgG AHA to NTZ IgG4 F(ab')$_{2pepsin}$; AHA4: IgG AHA to NTZ IgG4 F(ab')$_{2MMP\text{-}3}$; AHA5: IgG AHA to γ1 hinge peptide analogue cleaved by pepsin. AHA6: IgG AHA to γ1 hinge peptide analogue cleaved by MMP-3; AHA7: IgG AHA to γ4 hinge peptide analogue cleaved by pepsin; AHA8: IgG AHA to γ4 hinge peptide analogue cleaved by MMP-3.

Results

Baseline characteristics of early patients with SPRA, SNRA, and non-RA

The baseline characteristics of the patients with SPRA, SNRA, and non-RA are presented in Table I. Age, sex, smoking, symptom duration, and modified Health Assessment Questionnaire (mHAQ) did not differ significantly among the three groups. Conversely, the CDAI significantly differed among the three groups ($p<0.001$), and CDAI in SNRA ($p=5 \times 10^{-5}$) and SPRA ($p=1.5 \times 10^{-4}$) exhibited significantly higher than that in non-RA. Meanwhile, no significant differences were observed in CDAI between SNRA and SPRA, despite a higher tendency for SNRA. Serum CRP levels were significantly higher in SNRA and SPRA than those in non-RA ($p=0.002$ and $p=0.009$, respectively). Although the number of measurements for serum matrix metalloproteinase-3 (MMP-3), reflecting the activity of synovial involvement, was limited (SNRA, n=15; SPRA, n=26; non-RA, n=25), results comparable to those for CRP were obtained (SNRA *vs*. non-RA, $p=0.001$; SPRA *vs*. non-RA, $p=0.003$).

Comparing the serum levels of IgG AHAs in SPRA, SNRA, non-RA, and HC

Serum levels of IgG AHAs against eight distinct hinge antigens were compared among SPRA, SNRA, non-RA,

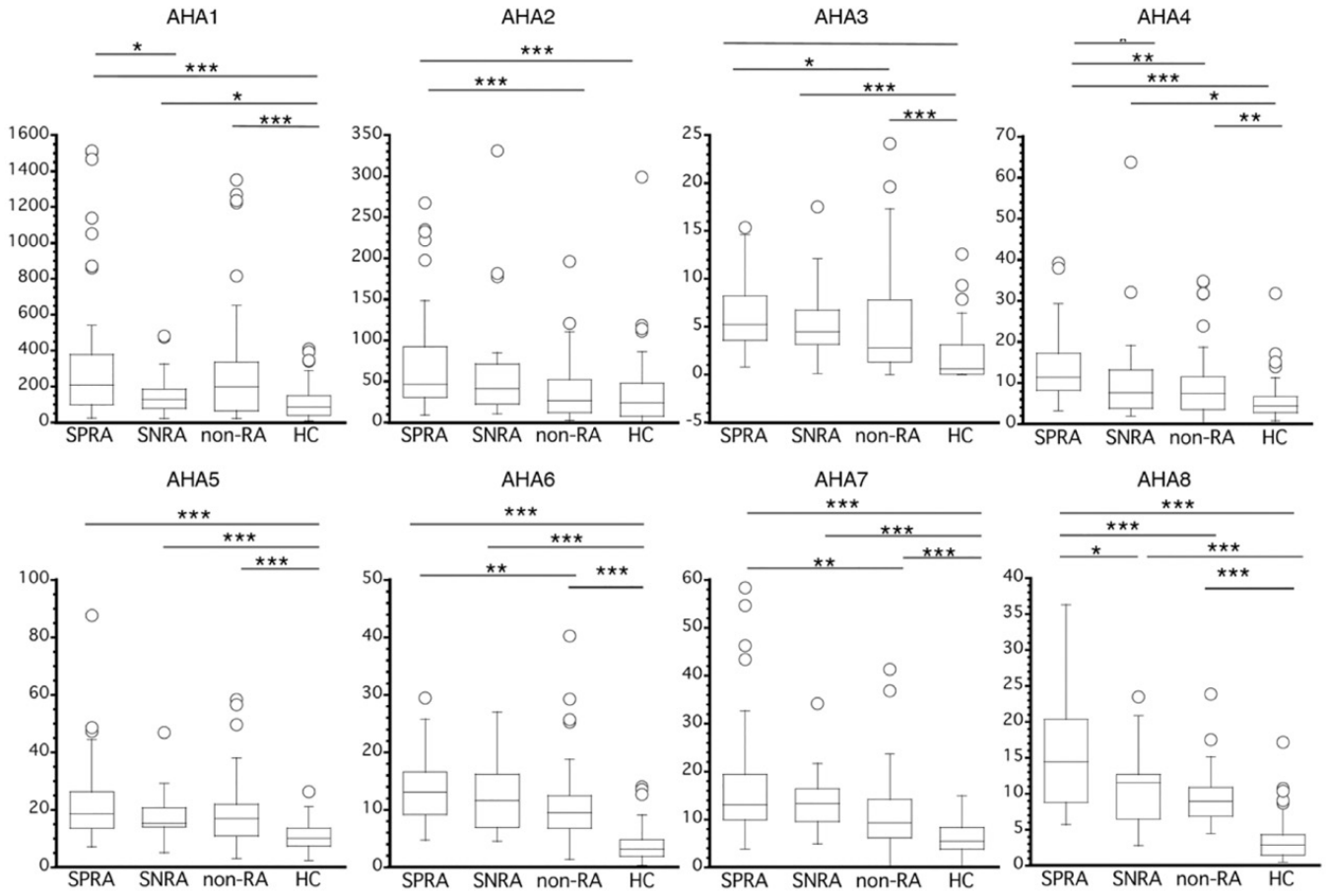

Fig. 2. Comparison of the AHA levels in SPRA (n=42), SNRA (n=21), non-RA (n=49) and HC (n=58). AHA1: IgG AHA to TCZ IgG1 F(ab')$_{2pepsin}$, AHA2: IgG AHA to TCZ IgG1 F(ab')$_{2MMP-3}$, AHA3: IgG AHA to NTZ IgG4 F(ab')$_{2pepsin}$, AHA4: IgG AHA to NTZ IgG4 F(ab')$_{2MMP-3}$, AHA5: IgG AHA to γ1 hinge peptide analogue cleaved by pepsin. AHA6: IgG AHA to γ1 hinge peptide analogue cleaved by MMP-3, AHA7: IgG AHA to γ4 hinge peptide analogue cleaved by pepsin, AHA8: IgG AHA to γ4 hinge peptide analogue cleaved by MMP-3. *p<0.05, ** p<0.01, *** p<0.001, TCZ: tocilizumab, NTZ: natalizumab.

and HC (Fig. 2). When compared to HC, SPRA showed significantly higher in all AHAs, whereas SNRA and non-RA exhibited significantly higher in seven AHAs, excluding AHA2. Comparisons between SPRA and SNRA revealed that SPRA was significantly higher in AHA1, AHA4, and AHA8. Comparisons between the SPRA and non-RA indicated significant differences in the six AHAs, except for AHA1 and AHA5. Comparisons between the SNRA and non-RA showed no statistically significant differences, although AHA7 levels were close to significance (AHA1: p=0.221, AHA2: p=0.132, AHA3: p=0.320, AHA4: p=0.734, AHA5: p=0.793, AHA6: p=0.160, AHA7: p=0.055, AHA8: p=0.132).

Logistic regression analyses for discrimination of SNRA from non-RA

The strength of the association between each variable (smoking, AHA1-AHA8, CRP, MMP-3, CDAI) in distinguishing SNRA from non-RA was assessed using unadjusted and adjusted univariate logistic regression analyses (Table II). The AUCs, indicating the utility of each variable for SNRA identification, ranged from 0.46 (AHA3) to 0.81 (MMP-3, CDAI) in the unadjusted analysis and from 0.59 (AHA4) to 0.84 (MMP-3) in the adjusted analysis, showing MMP-3 as the most useful. The odds ratios (ORs) for CRP, MMP-3, and CDAI were significantly higher in both analyses, suggesting their potential to discriminate SNRA from non-RA, although none of the AHAs were useful.

Next, we investigated whether combinations of CRP, MMP-3, CDAI, and AHAs could increase the AUCs. As multivariate logistic regression analyses had revealed that combinations of AHAs did not lead to an increase in AUCs (Fig. 3A), we investigated how CRP, MMP-3, and CDAI, in combination with AHAs, contribute to the increase in AUCs. Although combinations of CRP with AHAs could not increase AUCs, those of MMP-3 with two or more AHAs showed an increment of AUCs, such as the AUC (0.941) obtained from the combination with AHA7, AHA8, and MMP-3 (Fig. 3A and 3B). In addition, the AHA7 was selected along with MMP-3 for its significant association with SNRA (Table III). Finally, combinations of MMP-3, CDAI, AHA5, and AHA6 led to the maximum AUC (0.997) (Fig. 3A and C).

Comparison of predictive probabilities for SNRA

We calculated the predictive probabilities (p) of SNRA using the four models: Model 1 (sex, age, MMP-3), Model 2 (sex, age, AHA7, AHA8, MMP-3), Model 3 (sex, age, MMP-3, and CDAI), and Model 4 (sex, age, AHA5, AHA6, MMP-3, and CDAI).

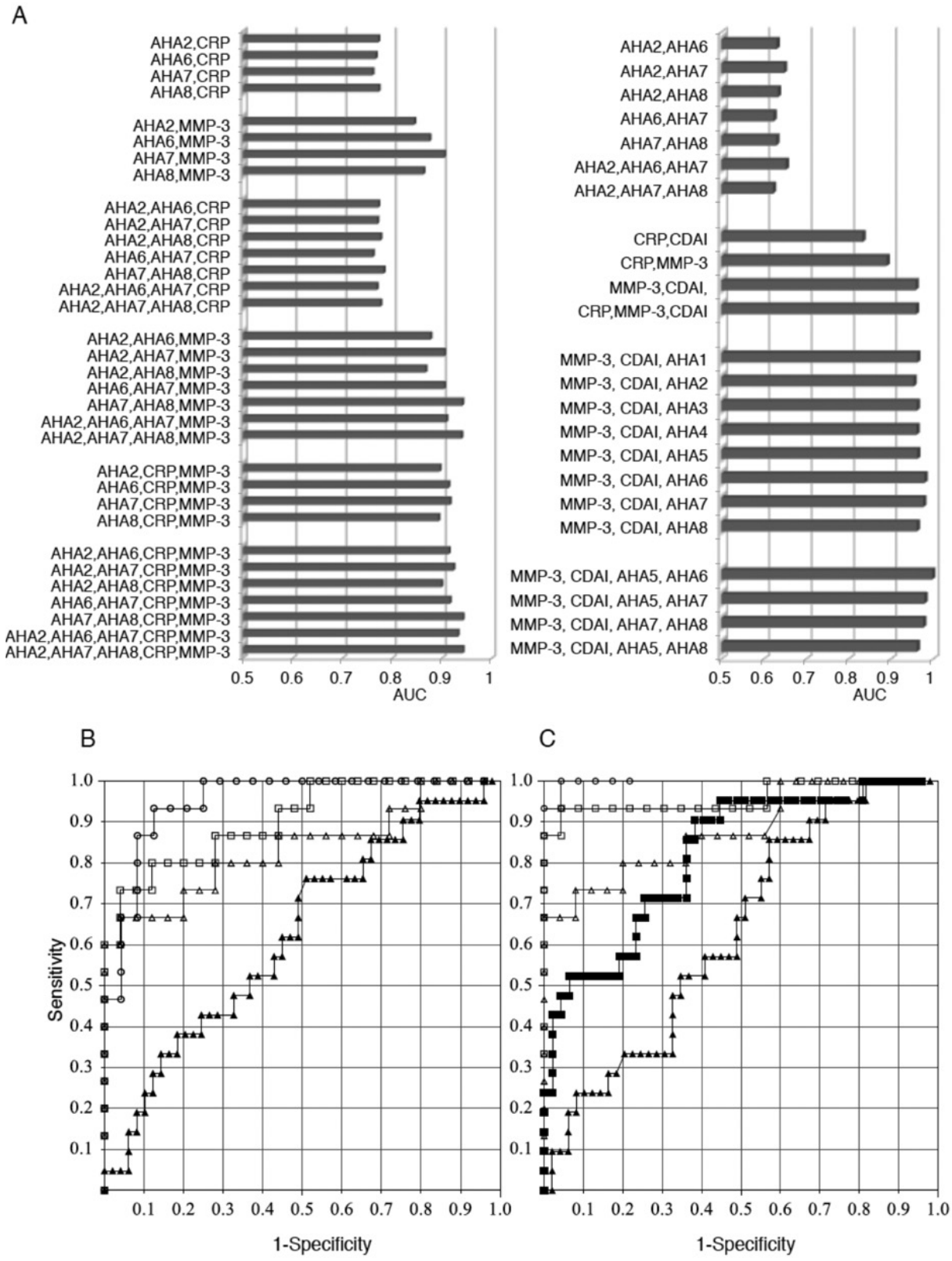

Fig. 3. Comparison of AUC for discriminating SNRA from non-RA.
A: AUC in combined variables obtained using logistic regression analyses (dependent variable: SNRA=1, non-RA=0).
B: ROC curves. AHA7 (closed triangle, AUC 0.62), MMP-3 (open triangle, AUC 0.84), combination of AHA7 and MMP-3 (open square, AUC 0.91), combination of AHA7, MMP-3, and AHA8 (open circle, AUC 0.94).
C: Combination of AHA5 and AHA6 (closed triangle, AUC 0.63), combination of AHA5, AHA6, and CDAI (closed square, AUC 0.82), combination of AHA5, AHA6, and MMP-3 (open triangle, AUC 0.88), combination of MMP-3 and CDAI (open square, AUC 0.96), combination of AHA5, AHA6, MMP-3, and CDAI (open circle, AUC 0.997).

Table III. Multivariate logistic regression analysis of the association between combined variables and SNRA when patients with non-RA were used as a control.

Variable	β	SE (β)	*p*	OR	95% CI
	1.406	2.687	0.601		
Age	-0.098	0.052	0.058	0.907	0.819-1.003
Sex	0.842	1.256	0.503	2.322	0.198-27.22
AHA7	0.306	0.148	**0.039**	**1.358**	**1.015-1.815**
AHA8	-0.245	0.225	0.276	0.783	0.504-1.215
MMP-3	0.023	0.009	**0.009**	**1.023**	**1.006-1.040**

The numbers of patients with SNRA and non-RA were 15 and 25, respectively. Patients with non-RA were osteoarthritis (n=7), polymyalgia rheumatica (n=5), unclassified arthritis (n=10), reactive arthritis (n=1), psoriatic arthritis (n=1), pustulotic arthro-osteitis (n=1). β: regression coefficient; SE: standard error; OR: odds ratio; 95% CI: 95% confidence interval.

The *p*-values for SNRA as the dependent variable were calculated using the following formula:

$$p=\frac{1}{1+e^{-X}}$$

$X=\beta_0+\beta_1x_1+\ldots\ldots+\beta_nx_n$

$\beta_0\sim\beta_n$: regression coefficients, $x_1\sim x_n$: independent variables

If a patient under consideration belongs to the SNRA, the *p* is approximately 1. By contrast, the *p* of a patient with non-RA is approximately 0. The distribution plots of the *p*-values derived from the models are shown in Fig. 4A. The Mann-Whitney U-test revealed significant differences between the SNRA and non-RA in all models ($p<0.01$). Comparing the four models in terms of accuracy, the Model 4 was the most accurate, followed by the Model 3, Model 2, and Model 1. Additionally, optimal cut-off values were obtained using ROC curve analyses, and various discriminatory properties were subsequently obtained using the chi-square test (Table IV). When comparing the discriminatory properties of the four models, the positive likelihood and odds ratios of the Model 3 and Model 4 were extremely high, suggesting their applicability in clinical practice.

To determine whether the same discriminatory accuracy can be achieved in established SNRA patients as well as early patients, of the patients with over 2 years' disease duration or DMARDs therapy before first visit as shown in Fig. 1, those with 2-year follow-up as SNRA (n=6) or arthritis other than RA (n=10) were evaluated (Fig. 4B). The Model 1 and 2 showed more variation in *p*-values and were inferior to the Model 3 and 4 in accuracy, whereas the Model 4 was the best, with a *p*-value of 1 in five of six SNRA cases and zero in five of eight non-RA cases. These results suggest the possibility of discriminating between SNRA and non-RA pa-

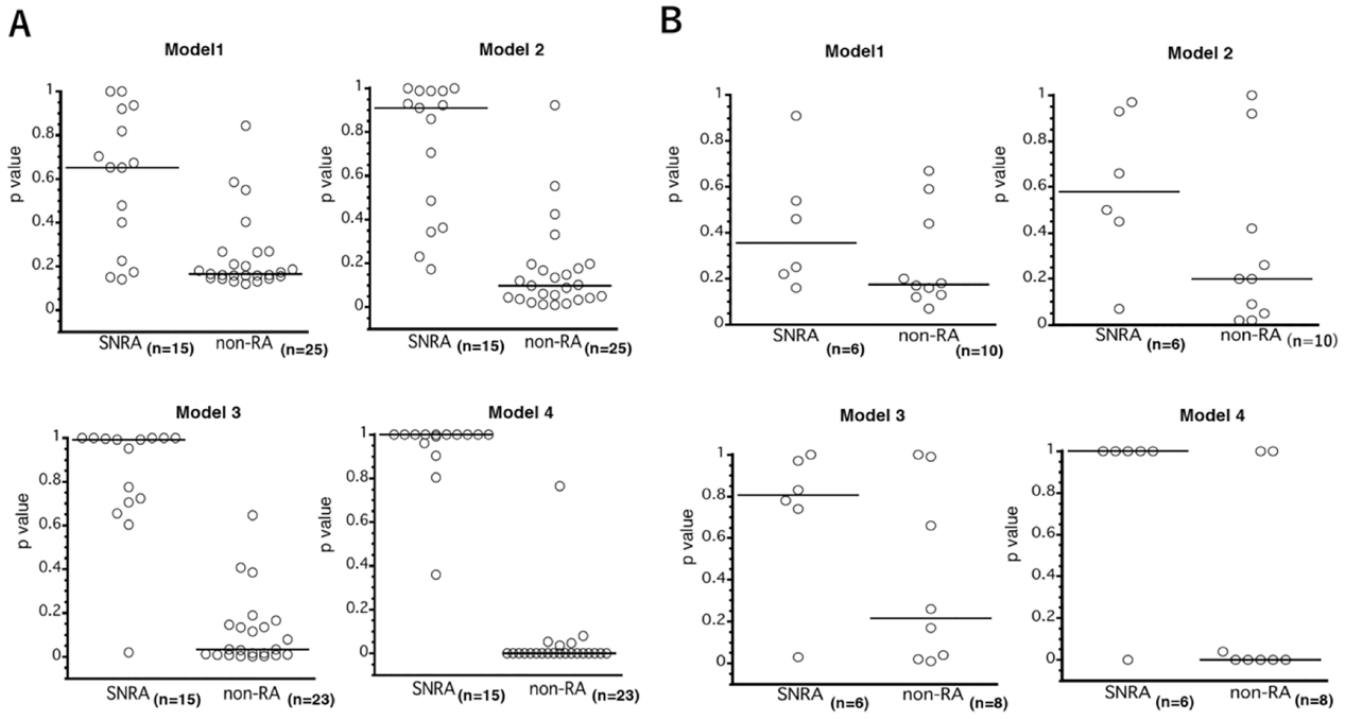

Fig. 4. Comparison of predicting probabilities (*p*) derived from various models between early patients with SNRA and non-RA (**A**) and established patients with SNRA and those with non-RA (**B**). Model 1 (sex, age, MMP-3), Model 2 (sex, age, AHA7, AHA8, MMP-3), Model 3 (sex, age, MMP-3, CDAI), Model 4 (sex, age, AHA5, AHA6, MMP-3, CDAI). The solid lines represent median value. The composition of early patients with non-RA in (A) was as follows: osteoarthritis (n=7), polymyalgia rheumatica (n=5), unclassified arthritis (n=10), reactive arthritis (n=1), psoriatic arthritis (n=1), pustulotic arthro-osteitis (n=1). An early non-RA patient with p-value of >0.5 in Model 4: a 56-year-old woman with persistent joint involvement (4-10 small joints) and negative RA-related autoantibodies had been followed up as UA. The composition of established patients with non-RA in (B) was as follows: osteoarthritis (n=2), polymyalgia rheumatic (n=3), pustulotic arthro-osteitis (n=2), psoriatic arthritis (n=1). Two established non-RA patients with *p*-value of 1 on Model 4 were psoriatic arthritis and pustulotic arthro-osteitis, respectively.

Table IV. The discriminatory properties for each model at optimum cut-off value.

Model (variables)	Cut-off	AUC (SE)	Sen/Spe	PPV/NPV	LR+ (95% CI)	OR (95% CI)
Model 1 (sex, age, MMP-3)	0.59	0.81 (0.08)	60/96	90/80	15.0 (7.1-31.6)	36.0 (6.1-212.1)
Model 2 (sex, age, MMP-3, AHA7, AHA8)	0.55	0.94 (0.03)	67/96	91/83	16.7 (7.3-37.8)	48.0 (8.2-280.6)
Model 3 (sex, age, MMP-3, CDAI)	0.41	0.96 (0.04)	93/96	93/96	21.5 (3.1-146.7)	308.0 (39.8-2386.3)
Model 4 (sex, age, MMP-3, CDAI, AHA5, AHA6)	0.36	0.997 (0.01)	93/96	93/96	21.5 (3.1-146.7)	308.0 (39.8-2386.3)

AUC: area under the curve; SE: standard error; Sen/Spec: sensitivity/specificity; PPV/NPV: positive predictive value/negative predictive value; LR+: positive likelihood ratio; 95% CI: 95% confidence interval; OR: odds ratio.
Composition of patients with non-RA: osteoarthritis (n=7), polymyalgia rheumatica (n=5), unclassified arthritis (n=10), reactive arthritis (n=1), psoriatic arthritis (n=1), pustulotic arthro-osteitis (n=1).

tients, irrespective of disease duration or DMARDs administration, if using Model 4.

Discussion

The concept of 'the window of opportunity' in RA, which involves diagnosing and initiating appropriate treatment within a very early period (<12 weeks) after the onset of joint symptoms, results in better outcomes by inhibiting the progression of RA inflammation (19, 20). To align with this concept, classification criteria incorporating a score emphasising RA-associated autoantibodies such as RF and anti-CCP have been developed (1) and used in clinical practice. In contrast, SNRA, which is negative for these two autoantibodies and has only a few affected joints, is considered more difficult and time-consuming to diagnose than SPRA (2, 3, 5). The main reason for the difficulty in diagnosing SNRA at an early phase is the lack of a biomarker such as an anti-CCP antibody. Before discussing the existence of a biomarker, the question arises as to whether SNRA is truly negative for all autoantibodies. Reed et al. pointed out that patients with SNRA who are negative for both IgM RF and IgG anti-CCP-2 antibodies are 9–30% positive for IgA RF, IgG RF, IgG anti-carbamylated protein, and various ACPA fine specificities, indicating that SNRAs are not autoantibody-free RA (21). As novel autoantibodies specific to SNRA have been discovered (22-25), SNRA may be considered an autoimmune disease with various autoantibodies. Recently, there has been a disease concept that RA is a continuum between SPRA, which tends toward an autoimmune mechanism, and SNRA, which tends toward an autoinflammatory mechanism (26), suggesting that we ought to reconsider the diagnosis, therapies, and management of RA.

IgG expresses a cryptic neoepitope in the upper or lower hinge across the core hinge region through the action of various proteolytic enzymes (27). Because autoantibodies against the hinge epitope do not react with native IgG, they are considered anti-modified protein antibodies and are collectively referred to as anti-hinge antibodies (AHA). Serum AHA has been detected at a high positivity rate and titer in some inflammatory autoimmune diseases, including RA, although its origin and clinical significance have not been fully elucidated (28). AHA has been considered a natural autoantibody because of its detection in the sera of healthy individuals to varying degrees and because it is generated by the antigen-inexperienced naive B cell compartment (11, 28). However, the serum AHA isotype predominantly belonged to the IgG and IgA classes, suggesting that it had undergone class-switch recombination (CSR). Furthermore, the sequences of complementarity determining region 3 (CDR3) in the heavy chain of IgG AHA-producing memory B cells revealed homologies ranging from 83.7–94%, indicating to be unreasonable to claim that AHA is a natural autoantibody (11). Mutated CDR3 sequences combined with CSR show that AHAs are produced as part of an active immune response (11).

With respect to clinical importance, IgG4 ACPA without complement activation has a relatively high incidence in SPRA, and the following aetiologic consequence has been proposed: IgG AHA restores complement activation by binding to cleaved IgG4, contributing to increased inflammation and joint destruction (17). Furthermore, elevated serum IgG $F(ab')_2$ and AHA levels in patients with inflammatory bowel disease (IBD) treated with TNF-neutralising agents are associated with a poor therapeutic response (29), suggesting that AHAs play some pathological role in chronic inflammatory autoimmune diseases. In contrast to the aforementioned autoimmune diseases, AHAs in infectious diseases and malignant tumours may exert favorable effects in organisms. Pathogenic microbes and tumor cells secrete proteases, such as IdeS from *Streptococcus pyogenes* and MMPs, respectively, and antibodies to pathogenic microbes and tumour cells are cleaved at the hinge region, followed by a reduction in effector functions such as complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP). *In vitro* studies have demonstrated that AHA can restore CDC, ADCC, and ADCP (11, 14, 17).

We investigated the discriminatory ability of AHA in patients with RA, mainly SPRA, treated with csDMARDs and bDMARDs (18). The significant association of IgG AHA to IgG4 $F(ab')_{2pepsin}$ with RA was observed not only in HC but also in non-RA patients, as controls. Moreover, the significant association between AHA against IgG4 $F(ab')_{2pepsin}$ and SNRA was also observed only when using HCs as control. In the present study, early-stage patients with DMARD-naive RA and non-RA were enrolled and analysed to determine whether there was an association between IgG AHA against IgG4 $F(ab')_{2pepsin}$ and SNRA. However, not every AHA in SNRA was significantly higher than that in non-RA, and logis-

tic regression analysis did not select any AHAs as independent variables for discriminating SNRA from non-RA. In contrast, the AUC for CRP, MMP-3, and CDAI using univariate logistic regression analyses were high, suggesting that the ability to differentiate between both diseases is higher than the AHA (Table II). Regarding the utility and position of serum MMP-3 in RA, serum MMP-3 levels are reportedly significantly higher in patients with RA than in healthy individuals, although no significant difference was observed between SNRA and SPRA (30). Additionally, serum MMP-3 levels in psoriatic arthritis where should be distinguished from SNRA, did not show significant difference from those in healthy subjects (31). Since these findings suggest that MMP-3 may be useful in distinguishing SNRA from non-RAs, it was inferred that the addition of AHAs to MMP-3 might further enhance its discriminatory ability. In fact, multivariate logistic regression analysis revealed that the Model 2 (AHA7, AHA8, MMP-3) showed high discriminatory ability (AUC = 0.94). In this study, we found the AHA7 was significantly associated with SNRA along with MMP-3, as shown in Table III. This perception again raises the possibility that AHAs against IgG4 hinge epitope, which appear under the action of pepsin, may have close association with RA, including SNRA. However, AHA against IgG4 $F(ab')_{2pepsin}$ (AHA3) showed no association with SNRA in this study. The cause is not clear but may reflect differences in characteristics between early-stage and DMARD-naive patients and established patients treated with DMARDs in the targeted SNRA population. Meanwhile, the specificity of AHA has been considered to be mainly located in the C-terminal amino acid sequence of the hinge as linear epitope (14). When using IgG4 $F(ab')_{2pepsin}$ as antigen, in addition to the linear epitope, it seems that the AHA recognises the conformational epitope.

The discriminatory ability (AUC=0.96) of the Model 3 (MMP-3, CDAI) was very high. To our knowledge, there are no reports about this discovery. The addition of AHA5 and AHA6 to the Model 3 led to highest discriminatory ability (Model 4, AUC=0.997), although it was unexpected that the hinge epitopes in IgG1 were selected instead of those in IgG4. Both models resulted in excellent discriminatory characteristics in clinical practice as shown in Table IV.

A limitation of this study is the lack of a validation study using another cohort consisting of early patients with SNRA and non-RA who were DMARD-naive. However, as shown in Fig 4B, the Model 4 resulted in similar results when established patients with SNRA and non-RA were used, suggesting the model possesses relatively high discriminatory ability between SNRA and non-RA regardless disease duration and/or DMARDs administration.

In conclusion, MMP-3 and CDAI alone were significantly associated with early SNRA, while AHA alone or in combination showed no significant association. However, combining MMP-3 with AHAs against IgG4 hinge peptide analogues improved the ability to discriminate SNRA from non-RA. Moreover, the combinatory model (AHAs against IgG1 hinge peptide analogues, MMP-3, CDAI) showed highest discriminatory ability. These findings suggest that AHAs are useful in discriminating between SNRA and non-RA.

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