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Clinical significance of anti-Ro52 (TRIM21) antibodies in adult patients with connective tissue diseases

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ABSTRACT

Objectives. Clinical significance of anti-Ro52 antibodies in connective tissue diseases (CTD) is controversial. Anti-Ro52 antibodies might be associated with a more severe CTD phenotype, especially interstitial lung disease (ILD). The aims of this study were to evaluate ILD prevalence and severity, the prevalence of micro- or macroangiopathy and CTD-associated cancers in CTD with anti-Ro52 antibodies.

Methods. CTD patients with anti-Ro52 antibody screening by immunoblot at diagnosis were enrolled. Two groups were retrospectively formed according to the presence of anti-Ro52 antibodies with an unbiased 1:1 matching on CTD types. Unsupervised multiple correspondence analysis and hierarchical clustering analysis were used to aggregate anti-Ro52 positive patients in subgroups.

Results. 408 CTD patients were included. Anti-Ro52 antibodies were detected in 33 % of CTD patients. Anti-Ro52 antibodies were associated with ILD at CTD diagnosis (47.8% vs. 23.0%, OR 3.3 95% IC 1.4 to 8.0, $p = 0.008$), even after adjusting for the presence of anti-Ro60 antibodies, especially in patients with antisynthetase syndrome, primary Sjögren syndrome and systemic sclerosis. Micro- or macroangiopathy was more frequent in anti-Ro52 positive CTD patients (18.6% vs. 9.7%, $p = 0.02$) and CTD patients with anti-Ro52 antibodies experienced more frequent relapses and required more immunosuppressive drugs. Clusters 4 and 5 identified anti-Ro52 positive CTD patients with severe ILD and with clinical features of systemic sclerosis or antisynthetase syndrome respectively.

Conclusions. We found that anti-Ro52 antibodies were independently associated with ILD in CTD patients irrespective of CTD type. Anti-Ro52 antibodies could be associated with severity and a more relapsing disease course in CTD patients.

Keywords: anti-Ro52 antibodies, connective tissue disease, interstitial lung disease

1. Introduction

Connective tissue diseases (CTD) – rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren syndrome (SS), systemic sclerosis (SSc), idiopathic inflammatory myopathies (IIM), mixed connective tissue disease (MCTD) – are a heterogeneous group of systemic autoimmune disorders. Like other autoimmune diseases CTD are characterized by the production of autoantibodies that can be detected in patients' sera. Most of the time, autoantibodies are used as diagnostic markers of CTD. Whether or not autoantibodies are involved in CTD pathogenesis is still debated. Anti-Ro/SSA antibodies are among the most commonly detected autoantibodies in the routine screening for autoimmune diseases [1]. Anti-Ro/SSA and anti-La/SSB antibodies are historically markers of SS and SLE. Anti-Ro/SSA and anti-La/SSB antibodies are directed against different proteins of intracellular small ribonucleic acid (RNA)-protein complexes. Two distinct proteins of the Ro/SSA complex encoded by different genes with no sequence homology are recognized by distinct autoantibodies: a 52-kDa protein and a 60-kDa protein [2,3]. Anti-Ro52 antibodies are also detected – most of the time in association with other autoantibodies – in IIM (30%) [4], SSc (20%) [5], auto-immune liver diseases (40%) and in some non-autoimmune conditions, such as cancers and viral infections [6–8]. Clinical relevance of anti-Ro52 antibodies in adult patients with CTD is controversial. Several studies have pointed out an association between anti-Ro52 antibodies and interstitial lung disease (ILD) in different CTD [5,9–13]. Patients with antisynthetase syndrome (ASS) and high titers of anti-Ro52 antibodies had more symptomatic ILD, more severe muscle and joint impairment [9]. Anti-Ro52 antibodies were associated with higher ILD prevalence in patients with SSc and MCTD [5,10]. Raynaud's phenomenon was more frequent in patients with juvenile myositis and anti-Ro52 antibodies [14]. Little is known about the prevalence of micro- or macroangiopathy in association with anti-Ro52 antibodies in adult CTD. Association between cancer occurrence and anti-Ro52 antibodies in CTD is unclear. So far, only one small size study has reported a higher prevalence of malignancies in patients with ASS and anti-Ro52 antibodies [9].

Therefore, the aims of this study were to precise ILD prevalence and severity, prevalence of micro- or macroangiopathy and prevalence of CTD-associated cancers in CTD patients with anti-Ro52 antibodies and to compare these clinical features in CTD with and without anti-Ro52 antibodies irrespective of CTD type.

2. Patients and methods

2.1. Patient selection

We conducted a retrospective multicentric matched case control study (NCT03565601) in 4 French tertiary care centers (Nancy, Lille, Dijon, and Metz). Study protocol was validated by the local ethics committee of Nancy University Hospital. Among the 2106 patients who had been screened for anti-Ro52 antibodies by immunoblot between January 2010 and December 2018 in immunology departments, patients with a new diagnosis of CTD and anti-Ro52 antibodies screening at the time of diagnosis were included (Fig. 1). All subjects were enrolled after providing informed consent. Then, two groups were retrospectively formed according to the presence (case) or not (control) of anti-Ro52 antibodies at diagnosis. An unbiased 1:1 matching on CTD types was performed.

2.2. Definitions of CTD

CTD were defined in accordance with updated international classification criteria as followed: 2010 ACR/EULAR criteria for RA [15], 2012 SLICC criteria for SLE [16], 2016 ACR/EULAR criteria for SS [17], 2013 ACR/EULAR criteria for SSc and 1975 Peter and Bohan criteria for IIM [18,19]. IIM were subdivided into ASS, dermatomyositis (DM), immune-mediated necrotizing myopathies (IMNM) and inclusion body myositis (IBM), based on clinical presentation, autoantibodies profile and muscle histopathologic features. MCTD were defined by fulfillment of at least one of the three followed criteria sets: modified Sharp's, Alarcon-Segovia or Kasukawa criteria [20-22]. Undifferentiated connective tissue diseases (UCTD) were defined by fulfillment of Mosca criteria [23]. In the case of multiple associated diseases, the main diagnosis was the one for which the patient was receiving specific care at the time of antibody testing.

2.3. Antibodies detection

Anti-Ro52 antibodies were detected in patients' sera at CTD diagnosis by line or dot immunoblot in all centers (EUROLINE Autoimmune Inflammatory Myopathies 16 Ag, Lubeck Germany; D-tek BlueDiver Dot Polymyositis/Scleroderma12 IgG, Mons Belgium) to standardize anti-Ro52 antibodies screening. Results were indicated as negative (0), weakly

positive (+), moderately positive (++) and strongly positive (+++). Other autoantibodies were detected at CTD diagnosis by usual local centers methods.

2.4. Data collection and definition

A standardized case report form captured demographic, clinical and laboratory features at the time of CTD diagnosis, 6 months after initial evaluation and at the time of the last follow-up. Specific treatments and relapses were also recorded. ILD presence at CTD diagnosis was defined by the identification of ILD signs on high-resolution computerized tomography (HRCT) interpreted by thoracic radiologists. HRCT patterns of ILD were determined based on conclusions of radiologists and/or clinicians in medical records. ILD severity at CTD diagnosis was assessed by NYHA dyspnea classification, pulmonary function tests – forced vital capacity (FVC), forced expiratory volume in the first second (FEV1) and diffusing capacity of the lung for carbon monoxide (DLCO) predicted values percentages – and 6-minute walk test (6MWT). Severe ILD was defined as FVC and/or FEV1 predicted values < 70% or DLCO predicted values < 50% at CTD diagnosis [24]. ILD worsening was defined as a decline > 10% of FVC and/or FEV1 predicted values or > 15% of DLCO predicted values [25]. Micro- or macroangiopathy was defined as digital ulcers, gangrene, or pulmonary arterial hypertension (PAH) occurrence during follow-up. Pulmonary arterial hypertension (PAH) was defined as a mean pulmonary arterial pressure (mPAP) \geq 20 mmHg, pulmonary artery occlusion pressure (PAOP) < 15 mmHg and pulmonary vascular resistance (PVR) > 3 Wood units on right heart catheterization (RHC) [26]. Only patients with suspected PAH by echocardiography had RHC. CTD-associated cancer was considered if malignancy occurred within the 2 years before or after CTD diagnosis. Relapse was defined as new specific CTD signs occurrence or worsening and/or new immunosuppressive or biologic agent initiation and/or a corticosteroid increase of at least 50% of the baseline dosage.

2.5. Statistical analysis

Characteristics of sample were described by percentage for categorical variables and mean and standard deviation for continued variables. McNemar's test for dichotomous variables and Wilcoxon signed-rank test for continuous variables were used to compare CTD patients with and without anti-Ro52 antibodies in paired comparison. Fisher's exact test for dichotomous variables and Student's t-test for continuous variables were used to compare

CTD patients with and without anti-Ro52 antibodies in unpaired comparison. Multivariate analysis used conditional logistic regression adjusted for anti-Ro60, anti-La and anti-Sm autoantibodies. Only variables with a p-value < 0.2 in univariate analysis were selected for the multivariate analysis. A multiple correspondence analysis (MCA) was performed in anti-Ro52 positive CTD patients. The table of inertia determined the number of axes to be retained. The hierarchical cluster analysis was performed on the number of dimensions retained in the MCA. The semi-partial R-square and the cubic clustering criterion were used to define the number of clusters to be selected. A description of the clusters was carried out to define profiles for each of them. Alpha risk was 5% for all analysis. Statistical analyses were performed using SAS 9.4 software (SAS Institute).

3. Results

3.1. Prevalence of CTD patients with anti-Ro52 antibodies

Among 2106 patients with anti-Ro52 antibodies screening we included 408 patients with both CTD and immunoblot testing at CTD diagnosis (Fig. 1). Anti-Ro52 antibodies were detected in 33% of CTD patients. Primary Sjögren syndrome (pSS) and ASS were associated with the higher prevalence of anti-Ro52 antibodies (57.1% and 59.7%, respectively). The prevalence of anti-Ro52 antibodies in all CTD types is described in online supplementary Table 1. Two hundred and twenty-six patients matched on CTD types were included in case-control analysis: 113 patients with anti-Ro52 antibodies and 113 patients without anti-Ro52 antibodies. No significant differences in gender, body mass index (BMI), tobacco consumption, age at diagnosis and follow-up duration were observed between CTD patients with and without anti-Ro52 antibodies (Table 1). As expected, associated Sjögren syndrome was more prevalent in CTD patients with anti-Ro52 antibodies compared to anti-Ro52 negative CTD patients (34.7% vs. 3.2%, $p < 0.0001$). Anti-Ro52 antibodies were associated with other specific autoantibodies in 83.6% of cases. Anti-Ro60 and anti-La autoantibodies were more frequent in CTD patients with anti-Ro52 antibodies. The prevalence of other autoantibodies was not significantly different among anti-Ro52 positive and negative CTD patients.

Table 1

General features of connective tissue disease (CTD) patients with and without anti-Ro52 antibodies.

	Total (N = 226)	Anti-Ro52 autoantibody negative (N = 113)	Anti-Ro52 autoantibody positive (N = 113)	P-value
Age at diagnosis	51.9 ± 16.4	51.2 ± 18.0	52.5 ± 14.6	0.7
Female	166/226 (73.4)	81/113 (71.7)	85/113 (75.2)	0.5
BMI	25.3 ± 5.0	25.5 ± 4.8	25.2 ± 5.2	0.09
Follow-up (months)	41.0 ± 26.5	42.1 ± 26.5	39.9 ± 26.6	0.6
Tobacco	88/226 (38.9)	48/113 (42.5)	40/113 (35.4)	0.2
CTD				
SLE	18/226 (8.0)	9/113 (8.0)	9/113 (8.0)	-
pSS	36/226 (15.9)	18/113 (15.9)	18/113 (15.9)	-
SSc	40/226 (17.7)	20/113 (17.7)	20/113 (17.7)	-
DM	22/226 (9.7)	11/113 (9.7)	11/113 (9.7)	-
ASS	58/226 (25.7)	29/113 (25.7)	29/113 (25.7)	-
IMNM	8/226 (3.5)	4/113 (3.5)	4/113 (3.5)	-
IBM	6/226 (2.7)	3/113 (2.7)	3/113 (2.7)	-
MCTD	12/226 (5.3)	6/113 (5.3)	6/113 (5.3)	-
UCTD	20/226 (8.8)	10/113 (8.8)	10/113 (8.8)	-
RA	6/226 (2.7)	3/113 (2.7)	3/113 (2.7)	-
Associated SS	36/190 (18.9)	3/95 (3.2)	33/95 (34.7)	<0.0001
Auto-antibodies				
Anti-Ro60	43/193 (22.3) ^a	10/101 (9.9) ^b	33/92 (35.9) ^b	<0.0001
Anti-La	25/226 (11.1)	1/113 (0.9)	24/113 (21.2)	<0.0001
Anti-dsDNA	22/226 (9.7)	12/113 (10.6)	10/113 (8.8)	0.3
Anti-Sm	8/226 (3.5)	7/113 (6.2)	1/113 (0.9)	0.03
Anti-RNP	33/226 (14.6)	13/113 (11.5)	20/113 (17.7)	0.09
ACA	13/226 (5.8)	8/113 (7.1)	5/113 (4.4)	0.3
Anti-Scl70	6/226 (2.7)	2/113 (1.8)	4/113 (3.5)	0.4
Anti-PmScl	22/226 (9.7)	13/113 (11.5)	9/113 (8.0)	0.3
Anti-aminoacyl tRNA synthetase	54/226 (23.9)	24/113 (21.2)	30/113 (27)	0.1
Anti-Ku	11/226 (4.9)	6/113 (5.3)	5/113 (3.5)	0.8
Anti-Mi2	10/226 (4.4)	6/113 (5.3)	4/113 (3.5)	0.5
Anti-p155/140 (TIF-1)	6/226 (2.7)	2/113 (1.8)	4/113 (3.5)	0.4
Anti-MDA5	9/226 (4.0)	6/113 (5.3)	3/113 (2.7)	0.3
Anti-HMGCR	1/214 (0.5) ^a	1/103 (1.0) ^b	0/111 (0.0) ^b	0.3
Anti-SRP	9/226 (4.0)	3/113 (2.7)	6/113 (5.3)	0.3

Dichotomous variables were represented as n/N (%) and continuous variables as mean ± SD. ^a N≠226 due to missing data. ^b N≠113 due to missing data. ACA, anticentromere antibodies; ASS, antisynthetase syndrome; BMI, body mass index; CTD, connective tissue disease; DM, dermatomyositis; dsDNA, doubled stranded deoxyribonucleic acid; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IBM, inclusion body myositis; IMNM, immune-mediated necrotizing myopathy; MCTD, mixed connective tissue disease; MDA5, melanoma differentiation associated protein-5; RA, rheumatoid arthritis; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SRP, signal recognition particle; pSS, primary Sjögren syndrome; SSc, systemic sclerosis; TIF-1, transcription intermediary factor 1; tRNA, transfer RNA; UCTD, undifferentiated connective tissue disease.

3.2. Interstitial lung disease (ILD) in anti-Ro52 positive CTD patients

Considering all CTD, we observed a higher prevalence of ILD in patients with anti-Ro52 antibodies in univariate analysis (47.8% vs. 23.0%, $p < 0.001$; Table 2). In multivariate analysis, anti-Ro52 antibodies were independently associated with ILD (OR 3.3, 95% IC 1.4 to 8, $p = 0.008$). When considering each CTD anti-Ro52 antibodies were associated with ILD in univariate analysis in pSS (27.8% vs. 0.0%, $p = 0.03$), SSc (55.0% vs. 25.0%, $p = 0.03$) and ASS (86.2% vs. 55.2%, $p = 0.02$). In multivariate analysis, anti-Ro52 antibodies were independently associated with ILD only in ASS (OR 4.0, 95% IC 1.1 to 14.2, $p = 0.03$). As expected, no case of ILD was reported in SLE, IMNM and IBM patients. ILD rarely occurred during follow-up in CTD patients. Radiological nonspecific interstitial pneumonia (NSIP), usual interstitial pneumonia (UIP) and organizing pneumonia (OP) patterns were found in 33/40 (82.5%), 5/40 (12.5%) and 2/40 (5%) patients, respectively. No difference of radiological ILD pattern was observed in CTD patients with and without anti-Ro52 antibodies (data not shown). In CTD associated ILD (CTD-ILD) anti-Ro52 negative patients were less symptomatic at the time of diagnosis (21.2% vs. 56.0% patients with functional NYHA class I, $p = 0.02$; Table 3). The proportions of severe ILD at diagnosis tended to be higher in CTD patients with anti-Ro52 antibodies, especially in antisynthetase syndrome. No significant difference in ILD severity was found between distinct anti-Ro52 positive CTD (data not shown).

Table 2

ILD prevalence in connective tissue disease (CTD) patients with and without anti-Ro52 antibodies.

	Total (N=226)	Anti-Ro52 autoantibody negative (N=113)	Anti-Ro52 autoantibody positive (N=113)	Univariate analysis P-value ^a	Multivariate analysis	
					OR (with 95% CI)	P-value
ILD at diagnosis						
All CTD	80/226 (35.4)	26/113 (23.0)	54/113 (47.8)	< 0.0001	3.3 (1.4-8.0)	0.008
SLE	0/18 (0.0)	0/9 (0.0)	0/9 (0.0)	1.0	-	-
pSS	5/36 (13.9)	0/18 (0.0)	5/18 (27.8)	0.03	15.1 (0.6-389.4)	0.1
SSc	16/40 (40.0)	5/20 (25.0)	11/20 (55.0)	0.03	4.1 (0.9-17.2)	0.05
DM	5/22 (22.7)	2/11 (18.1)	3/11 (27.3)	0.7	-	-
ASS	41/58 (70.7)	16/29 (55.2)	25/29 (86.2)	0.02	4 (1.1-14.2)	0.03
IMNM	0/8 (0.0)	0/4 (0.0)	0/4 (0.0)	-	-	-
IBM	0/6 (0.0)	0/3 (0.0)	0/3 (0.0)	-	-	-
MCTD	7/12 (58.3)	2/6 (33.3)	5/6 (83.3)	0.2	-	-
UCTD	5/20 (25.0)	1/10 (10.0)	4/10 (40.0)	0.2	-	-
RA	1/6 (16.7)	0/3 (0.0)	1/3 (33.3)	-	-	-
ILD during follow-up						
All CTD	83/226 (36.7)	28/113 (24.8)	55/113 (48.7)	< 0.0001	3.8 (1.5-9.8)	0.005

Dichotomous variables were represented as n/N (%). ^a Only CTD subgroups with more than 5 patients were analyzed. ASS, antisynthetase syndrome; DM, dermatomyositis; IBM, inclusion body myositis; IC: confidence interval; IMNM, immune-mediated necrotizing myopathy; ILD: interstitial lung disease; MCTD, mixed connective tissue disease; OR, odds ratio; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; pSS, primary Sjögren syndrome; SSc, systemic sclerosis; UCTD, undifferentiated connective tissue disease.

Table 3

ILD severity in connective tissue disease (CTD) patients with and without anti-Ro52 antibodies.

	Total (N=80)	Anti-Ro52 autoantibody negative (N=26)	Anti-Ro52 autoantibody positive (N=54)	P-value
Dyspnea at diagnosis (NYHA class)				0.02
I	25/77 (32.5) ^a	14/25 (56.0) ^b	11/52 (21.2) ^c	
II	19/77 (24.7) ^a	3/25 (12.0) ^b	16/52 (30.8) ^c	
III	24/77 (31.2) ^a	5/25 (20.0) ^b	19/52 (36.5) ^c	
IV	9/77 (11.7) ^a	3/25 (12.0) ^b	6/52 (11.5) ^c	
Severe ILD at diagnosis				0.08
All CTD	39/80 (48.8)	9/26 (34.6)	30/54 (55.6)	
pSS	2/5 (40)	-	2/5 (40)	-
SSc	10/17 (58.8)	3/6 (50)	7/11 (63.6)	0.6
DM	1/5 (20)	0/2 (0)	1/3 (33.3)	1.0
ASS	20/39 (51.3)	5/15 (33.3)	15/24 (62.5)	0.1
MCTD	3/7 (42.9)	0/2 (0)	3/5 (60)	0.4
UCTD	2/5 (40)	1/1 (100)	1/4 (25)	0.4
FVC at diagnosis (% predicted value)	79 ± 21	82 ± 24	78 ± 20	0.4
FEV1 at diagnosis (% predicted value)	78 ± 20	83 ± 22	76 ± 19	0.2
DLCO at diagnosis (% predicted value)	55 ± 17	56 ± 15	55 ± 18	0.7
6MWT at diagnosis (% predicted value)	70 ± 20	68 ± 18	71 ± 21	0.7
Worsening ILD	9/69 (13.0) ^a	2/26 (7.7)	7/43 (16.3) ^c	0.5

Dichotomous variables were represented as n/N (%), continuous variables as mean ± SD. ^a N≠80 due to missing data. ^b N≠26 due to missing data. ^c N≠54 due to missing data. ASS, antisynthetase syndrome; CTD, connective tissue disease; DLCO, diffusing capacity for carbon monoxide; DM, dermatomyositis; ILD, interstitial lung disease; FEV1, forced expiratory volume; FVC, functional vital capacity; MCTD, mixed connective tissue disease; pSS, primary Sjögren syndrome; SSc, systemic sclerosis; UCTD, undifferentiated connective tissue disease; 6MWT, 6-minute walk test.

3.3. Other clinical manifestations and disease course in anti-Ro52 positive CTD patients

In univariate analysis, micro- or macroangiopathy was more prevalent in patients with anti-Ro52 antibodies irrespective of CTD type (18.6% vs. 9.7%, $p = 0.02$; Table 4). Among 32 patients with micro- or macroangiopathy, 23 patients had SSc, 3 patients had DM, 3 patients had ASS, 2 patients had MCTD and one patient had UCTD (data not shown). Raynaud's phenomenon frequency was similar in CTD patients with and without anti-Ro52 antibodies. No difference was observed regarding frequencies of articular manifestations and the prevalence of CTD-associated cancers in anti-Ro52 positive and negative patients. Among 226 patients 68 relapses were observed in 52 patients. In univariate analysis, relapses were more frequent in CTD patients with anti-Ro52 antibodies (29.2% vs. 16.8% patients with at least one relapse during follow-up, $p = 0.04$; Table 4). CTD patients with anti-Ro52 antibodies received more immunosuppressants than patients without anti-Ro52 antibodies (2.5 vs. 1.8, $p = 0.0002$). The proportion of CTD patients receiving 3 or more immunosuppressive drugs during follow-up was higher in anti-Ro52 positive patients (47.8% vs. 31.0%, $p = 0.005$). Anti-Ro52 positive patients were given more corticosteroids, cyclophosphamide, rituximab, intravenous immunoglobulins and other disease-modifying antirheumatic drugs (DMARDs).

Table 4

Extra-pulmonary manifestations prevalence, disease outcomes and medications used in connective tissue disease (CTD) patients with and without anti-Ro52 antibodies.

	Total (N=226)	Anti-Ro52 autoantibody negative (N=113)	Anti-Ro52 autoantibody positive (N=113)	Univariate analysis p value	Multivariate analysis		D i c h o t o m o u s v a r i a b l e s w e r e
					OR (with 95% CI)	P-value	
Raynaud's phenomenon	107/226 (47.3)	51/113 (45.1)	56/113 (49.6)	0.5	-	-	
Micro- or macroangiopathy	32/226 (14.2)	11/113 (9.7)	21/113 (18.6)	0.02	2 (0.6-6.5)	0.3	
Digital tip ulcers	24/226 (10.6)	9/113 (8.0)	15/113 (13.3)	0.1	-	-	
Digital ischemia	7/226 (3.1)	2/113 (1.8)	5/113 (4.4)	0.3	-	-	
PAH	3/226 (1.3)	1/113 (0.9)	2/113 (1.8)	1	-	-	
Arthralgia	122/226 (54.0)	61/113 (54.0)	61/113 (54.0)	1	-	-	
Arthritis	58/226 (25.7)	31/113 (27.4)	27/113 (23.9)	0.5	-	-	
CTD-associated cancer	18/226 (8.0)	8/113 (7.1)	10/113 (8.8)	0.6	-	-	
Number of relapses	0.3 (0.6)	0.2 (0.5)	0.4 (0.7)	0.04	-	-	
Patients with ≥ 1 relapse	52/226 (23.0)	19/113 (16.8)	33/113 (29.2)	0.04	1.6 (0.7-3.5)	0.3	
Number of immunosuppressants used	2.2 (1.5)	1.8 (1.3)	2.5 (1.6)	0.0002	-	-	
Patients with ≥ 3 immunosuppressants used	89/226 (39.4)	35/113 (31.0)	54/113 (47.8)	0.005	1.6 (0.7-3.6)	0.2	
Medications received							
Oral/IV steroids	162/226 (71.7)	75/113 (66.4)	87/113 (77.0)	0.05	1.3 (0.5-3.1)	0.6	
Cyclophosphamide	27/226 (11.9)	6/113 (5.3)	21/113 (18.6)	0.004	2.7 (0.8-8.9)	0.1	
Other DMARDs ^a	122/226 (54.0)	53/113 (46.9)	69/113 (61.1)	0.02	1.5 (0.7-3.3)	0.3	
Rituximab	21/226 (9.3)	5/113 (4.4)	16/113 (14.2)	0.01	1.9 (0.5-7.6)	0.4	
IV immunoglobulin	38/226 (16.8)	12/113 (10.6)	26/113 (23.0)	0.006	2.7 (0.9-8.3)	0.08	

represented as n/N (%) and continuous variables as mean \pm SD. ^a Methotrexate, Azathioprine, Mycophenolate mofetil, Cyclosporine, Tacrolimus. DMARDs, disease-modifying anti-rheumatic drugs; PAH, pulmonary arterial hypertension. IV, intravenous.

3.4. Hierarchical clustering analysis in CTD patients with anti-Ro52 antibodies

Twenty-eight binary variables were selected for hierarchical clustering analysis according to their clinical relevance. One hundred and four anti-Ro52 positive CTD patients with complete data for the 28 selected variables were included for the MCA. We performed MCA on the first 4 dimensions (cumulatively explaining 40.3% of the variance; online supplementary Fig. 1) and then hierarchical clustering to identify clusters (Fig. 2). The hierarchical tree suggested a partition into 6 clusters. Twenty-two variables were discriminant for the hierarchical cluster analysis. Patients' characteristics among the 6 identified clusters were described in online supplementary Table 2. In the first cluster, patients were older at the time of diagnosis and no muscular involvement was reported. Most of patients experienced no relapse. Patients of cluster 2 were younger and had arthritis. Anti-Ro60 and anti-La antibodies positivity was frequent. Myositis, Raynaud's phenomenon and digital tip ulcers were uncommon. Patients of cluster 3 had mechanic hands, articular manifestations and antisynthetase antibodies. Corticosteroids and DMARDs were frequently used. No patient experienced relapse in cluster 3. In the fourth cluster patients had severe ILD at diagnosis and vascular manifestations (Raynaud's phenomenon, digital tip ulcers). The presence of anti-Ro60, anti-La and anti-RNP antibodies was common. Patients received more immunosuppressants during follow-up: all patients received corticosteroids and DMARDs; cyclophosphamide or rituximab were frequently given. Patients of cluster 5 experienced arthralgia, pericarditis, and myositis. Most patients had antisynthetase antibodies. As in cluster 4, patients had severe ILD at diagnosis and received more immunosuppressive drugs. Relapses were frequent. In the sixth cluster patients were older, had cutaneous signs of DM and myositis. CTD-associated cancers were frequently observed. Corticosteroids, DMARDs and immunoglobulins were repeatedly used.

3.5. Comparison of clinical manifestations and disease course between anti-Ro52⁺anti-Ro60⁻ and anti-Ro52⁺anti-Ro60⁺ CTD patients

We aimed to study the impact of anti-Ro52 and anti-Ro60 antibodies association on the prevalence of clinical manifestations. Anti-Ro60 antibodies status was available in 92 anti-Ro52 positive patients. We compared the previously described clinical manifestations in online supplementary Table 3. We observed a non-significant higher ILD prevalence at diagnosis and a higher frequency of symptomatic ILD patients at diagnosis in anti-Ro52⁺antiRo60⁻ CTD patients. Prevalence of micro- or macroangiopathy, prevalence of CTD-associated cancers, number of relapses, number of immunosuppressants taken were similar in anti-Ro52 positive CTD patients with and without associated anti-Ro60 antibodies.

3.6. Phenotype of CTD patients with isolated anti-Ro52 antibodies

Fourteen CTD patients had isolated anti-Ro52 antibodies without any other specificities: 7 patients with SSc, 5 patients with SS and 2 patients with ASS. ILD at diagnosis was found in 7/14 (50%) patients. Severe ILD was observed in 5/8 (63%) patients. No case of worsening ILD was reported. Raynaud's phenomenon was present in 8/14 (57%) patients and micro- or macroangiopathy occurred in 5/14 (36%) patients. Two patients with SSc developed PAH. One case of CTD-associated cancer was observed. Among patients with isolated anti-Ro52 antibodies, 4/14 (29%) patients had at least one relapse during follow-up and 4/14 (29 %) patients used 3 or more immunosuppressive drugs.

4. Discussion

In this study, we used a multicentric cohort of CTD patients to study the clinical significance of anti-Ro52 antibodies in CTD. We found that anti-Ro52 antibodies were strongly associated with ILD at the time of diagnosis in CTD patients irrespective of CTD type. We reported no association between anti-Ro52 antibodies and CTD-associated cancer.

Importantly, the presence of anti-Ro52 antibodies was still associated with ILD at diagnosis even after adjusting for the coexistence of other autoantibodies, especially anti-Ro60 antibody, one of the most associated antibodies with anti-Ro52 antibody. Although non-significant, ILD prevalence at CTD diagnosis was higher in anti-Ro52⁺ anti-Ro60⁻ patients compared to anti-Ro52⁺ anti-Ro60⁺ patients, supporting previously reported data by Wu *et al* [27]. Our findings regarding anti-Ro52 antibody and ILD association confirm previously reported data in various CTD such as pSS, SSc, ASS and MCTD separately [5,9–13]. In our subgroups analyses ILD was significantly more frequent in pSS, SSc and ASS with anti-Ro52 antibodies. A similar tendency was noted in patients with MCTD and UCTD without reaching the statistical significance, probably because of the small number of patients in these subgroups. However, no ILD was noticed in patients with SLE, IBM or IMNM. Even if this result needs to be confirmed in a larger cohort the association of anti-Ro52 antibodies and ILD seemed to concern only CTD, for which ILD is a usual manifestation.

NSIP was the most frequent HRCT pattern described in CTD patients (more than 80 % of patients) confirming previously reported data [28]. In our study, the high proportion of NSIP was probably explain by the high number of patients with ASS and the low number of patients with RA.

Anti-Ro52 antibodies have been associated with more severe and refractory disease in juvenile myositis and in adult ASS [9,11,14,29]. In our cohort, CTD patients without anti-Ro52 antibodies were less symptomatic at diagnosis. We observed a strong tendency to more severe ILD –by pulmonary function tests – at the time of diagnosis in anti-Ro52 positive CTD patients. In univariate analysis, anti-Ro52 antibodies were also associated with more relapses and immunosuppressants used during follow-up in CTD patients. The higher proportions of patients who received corticosteroids, DMARDs, including cyclophosphamide, rituximab, and intravenous immunoglobulins, could reflect disease severity and relapsing course in anti-Ro52 positive CTD. Our findings suggest that anti-Ro52 antibody positivity could represent a marker of CTD severity – especially ILD severity – and might be a predictor of poor CTD outcome.

Micro- and macroangiopathy are rare in CTD patients. In our study, we used a composite criterion reflecting the severity of vascular manifestations and including digital tip ulcers, distal ischemia, and PAH. In univariate analysis, micro- or macroangiopathy was more prevalent in anti-Ro52 positive CTD patients. As expected, micro- or macroangiopathy occurred mostly in patients with SSc. However, this is the first report of an association between anti-Ro52 antibodies and micro- or macroangiopathy. This point needs clearing up by additional studies.

In hierarchical clustering analysis, we identified 6 clusters in anti-Ro52 positive CTD patients. Clusters 3 and 5 seem to correspond to patients with antisynthetase syndrome, while cluster 6 appears to represent patients with dermatomyositis. Cluster 4 identified a group of patients with vascular phenotype (Raynaud's phenomenon, digital tip ulcers) probably corresponding to patients with systemic sclerosis. Interestingly, patients in clusters 4 and 5 had severe ILD at diagnosis, experienced more frequent relapses and received more immunosuppressive drugs during follow-up. This unsupervised analysis confirms that anti-Ro52 antibodies are associated with severe pulmonary manifestations in patients with antisynthetase syndrome and systemic sclerosis.

The association of anti-Ro52 antibodies with higher CTD-ILD frequency and with a more severe CTD phenotype remains to be elucidated. Ro52 is one of the most highly antigenic self-proteins [30]. Ro-52 reactivity could represent an epiphenomenon in tissues targeted by the immune response, especially in lungs; Ro52 reactivity could be related to a strong immune response and subsequent tissues damages reflecting clinical severity. The fact that we found no correlation between clinical manifestations and anti-Ro52 antibody titers supports this hypothesis (data not shown).

Our study has several limitations. Even though we used a large cohort of CTD patient, some epidemiologically prevalent CTD types – such RA and SLE – are poorly represented in subgroups analyses. Our CTD cohort is not a representative sample of anti-Ro52 positive CTD patients, as anti-Ro52 antibodies are not tested routinely in RA and SLE in all centers, explaining their low prevalence in our study. To standardize anti-Ro52 antibodies detection in all centers, we only included CTD patients, for whom an immunoblot technique was used, resulting in an obvious selection bias towards CTD with muscular manifestations. The association of anti-Ro52 antibodies with vascular manifestations, more relapses and more immunosuppressive drugs used were lost in multivariate analysis, thus we cannot exclude potential confounders. In our cohort, data was collected retrospectively according to defined precise evaluation points (time of diagnosis, 6 months after initial evaluation) for appropriate

comparisons of functional NYHA class and pulmonary function tests data. This resulted in some missing data. Immunoblot technique lacks inter-test reproducibility and false positive results are common [31]. However, the prevalence of anti-Ro52 antibodies in CTD was very similar in previous studies [32]. In case of associated CTD, only the main diagnosis was taken into account. Therefore, the impact of associated Sjögren syndrome in CTD patients could not be evaluated. For hierarchical clustering analysis we had to exclude various discriminant CTD variables, such as specific clinical manifestations and autoantibodies because of statistical discrepancies, leading to an incomplete description of aforementioned clusters.

To conclude, we found that anti-Ro52 antibodies were independently associated with interstitial lung disease prevalence at CTD diagnosis (almost half of anti-Ro52 positive CTD patients suffered from ILD at diagnosis), even after adjusting for the coexistence of anti-Ro60 antibodies. Currently, anti-Ro52 antibody testing is still a matter of debate among immunologists and clinicians. If anti-Ro52 antibodies are not suitable biological markers for CTD diagnosis, our results suggest a potential use of anti-Ro52 antibodies as a CTD-ILD predictor. Anti-Ro52 antibody positivity should lead clinicians to a careful screening of ILD – a life-threatening complication – at CTD diagnosis, particularly in ASS, SSc and pSS. According to our findings, anti-Ro52 antibody testing could be integrated into evaluation, monitoring and management of patients with CTD in the future.

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Figure legend

Fig. 1. Flow chart of CTD patients included in 1:1 case-control analysis. Abs, antibodies; CTD, connective tissue disease.

Fig. 2. Dendrogram and hierarchical cluster analysis in anti-Ro52 positive CTD patients. The y-axis indicates the height of fusion into clusters proposed and the x-axis indicates CTD patients (n = 104). A hierarchical tree indicates CTD patients according to the cluster that they belong to. CTD, connective tissue disease.

Supplementary Fig. 1. Multiple correspondence analysis factor maps in anti-Ro52 positive CTD patients. Factors maps showing raw data (patients) used to generate the dendrogram. Dimensions 1 and 2 cumulatively explained 24.7% of the total variance. Dimensions 1 and 3 cumulatively explained 23.5% of the total variance. Dimensions 1 and 4 cumulatively explained 22.0% of the total variance. Dimensions 2 and 3 cumulatively explained 18.2% of the total variance. Dimensions 2 and 4 cumulatively explained 16.7% of the total variance. Dimensions 3 and 4 cumulatively explained 15.5% of the total variance. CTD, connective tissue disease.



