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The importance of detecting anti-DFS70 in routine clinical practice: comparison of different care settings

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Abstract

Background: Screening for antinuclear antibodies by indirect immunofluorescence (ANA-IIF) is essential in the diagnostic workup of ANA-associated autoimmune rheumatic diseases (AARDs). However, also healthy individuals may test positive, making the interpretation challenging. Recent reports suggest that dense fine speckled 70 antibodies (anti-DFS70) may facilitate this challenge. Here, we investigate their clinical importance based on data from four Belgian laboratories (one primary, two secondary and one tertiary care).

Methods: At least one specific DFS70 assay (DFS70 IgG ELISA or lineblot [Euroimmun, full length antigen] and/or DFS70 IgG CLIA [Inova Diagnostics, truncated antigen]) was performed on four consecutive cohorts of homogeneous-like ANA-IIF samples ($n=697$). Co-occurrence with AARD-specific ANA and clinical information were documented in the anti-DFS70-positive samples.

Results: Using a combination of solid phase techniques, we found between 7.6% and 26% anti-DFS70 in the

different cohorts. Focusing on anti-DFS70 CLIA-positive samples without co-occurrence of AARD-specific ANA, we observed a trend towards lower frequency in tertiary (8% [$p=0.0786$]) and secondary care (12% [$p=0.1275$] and 6% [$p<0.001$]) compared to primary care (21%). Moreover, in this specific subpopulation, AARD was less frequent (0%–50% compared to 6%–77% in the total anti-DFS70-positive group).

Conclusions: Anti-DFS70 prevalence depends on the applied assay and care setting. Our data suggest that, for an ANA-IIF-positive patient, it is rather the absence of AARD-associated ANA and clinical symptoms that contribute to the exclusion of AARD than the presence of anti-DFS70. Nevertheless, isolated anti-DFS70 helps to clarify positive ANA-IIF results, especially if pretest probability for AARD is low.

Keywords: anti-DFS70/LEDGF; antinuclear antibodies; systemic rheumatic autoimmune diseases.

Introduction

Antinuclear antibody (ANA) detection is considered essential in the diagnostic workup of ANA-associated autoimmune rheumatic diseases (AARDs) [1, 2]. In the routine laboratory, indirect immunofluorescence (IIF) on HEp-2(000) cells is the most widely applied screening method [3]. However, because screening for ANA by indirect immunofluorescence (ANA-IIF) lacks specificity with up to 20% positivity found in apparently healthy individuals, further testing to identify the specific antigenic targets of ANA is essential to improve the diagnostic contribution of the positive ANA-IIF test result [4].

Clinicians may have different reasons for requesting ANA tests. ANA results can be used to support or to assist the clinical diagnosis in patients with symptoms of AARD. In addition, in some AARD (e.g. systemic sclerosis), the presence of specific ANA is also associated with particular disease manifestations emphasizing their use as prognostic markers [5]. However, in daily clinical practice, an evolution in the request pattern is observed with ANA tests

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more frequently ordered in a wider clinical framework. In this context with low pretest probability, a negative ANA-IIF test may contribute to exclude some AARDs like systemic lupus erythematosus, mixed connective tissue disease and systemic sclerosis [4]. Nevertheless, this approach also results in a higher frequency of ‘clinically out of context’ ANA-IIF positivity, leading to unnecessary follow-up testing, possible misdiagnosis and even incorrect therapies [1]. Recent observations suggest that isolated DFS70 antibodies (anti-DFS70) might contribute to explaining a significant proportion of these AARD-unrelated ANA-IIF-positive results [6].

Anti-DFS70 target the lens epithelium-derived growth factor (LEDGF) and react with a conserved and conformational epitope, restricted to a single region located within the integrase binding domain at the C-terminal of the protein [7]. On ANA-IIF, anti-DFS70 are characterized by a dense fine speckled (DFS) staining of the nucleoplasm of the interphase cells, typically excluding the nucleoli, and by a strong granular-speckled staining of the condensed mitotic chromosomes (for reference images, see website of ICAP [International consensus on Antinuclear Antibody Pattern, <http://www.anapatterns.org>], pattern AC-2) [8, 9].

Anti-DFS70/LEDGF were first described in 1994 in interstitial cystitis and later on in a variety of other non-autoimmune conditions (e.g. atopic dermatitis, asthma, gynecologic syndromes and neoplasia), autoimmune conditions (e.g. autoimmune thyroiditis and AARD such as Sjögren’s syndrome and systemic lupus erythematosus) and even healthy donors [7, 10–16].

Several studies observed higher frequencies of anti-DFS70 in healthy individuals compared to AARD [2, 12, 15, 17, 18]. In a recent meta-analysis of five studies, a mean prevalence of $7.8\% \pm 6.2\%$ (median 7.6%) anti-DFS70 in healthy controls was calculated, regardless the detection method used. In the same study, the calculated mean prevalence of anti-DFS70 in AARD was $4.5 \pm 2.8\%$ (median 3.9%), with isolated anti-DFS70 reactivity being extremely rare ($0.7 \pm 0.9\%$ [median 0.45%]) [7]. Based on these findings, some authors suggested that isolated anti-DFS70 positivity could be used to exclude the diagnosis of AARD [2, 7]. By contrast, for other authors, this proposal is difficult to support as the ANA test is usually not requested in healthy individuals, resulting in a substantial part of the anti-DFS70-positive tests in patients with AARD [19]. Their idea was also supported by the limited data available on unselected sera for which routine ANA screening was requested, showing low prevalence of confirmed anti-DFS70 ($1.7\% \pm 0.1\%$ [median 1.7%]), approaching the situation observed in AARD [2, 7].

Various other factors contribute to the controversy on the true clinical significance of anti-DFS70. First, differences in frequencies of anti-DFS70 are observed depending on the detection technique (ANA-IIF, enzyme-linked sorbent immunoassay [ELISA], chemiluminescence immunoassay [CLIA] or immunoblot) used. For instance, Bizzaro and coworkers demonstrated that only 14% of their initial DFS-ANA-IIF-positive sera, were positive on the DFS70 ELISA. In addition, they also observed important HEP-2 cell line variability [19]. Second, differences between the solid phase assays were also described, probably related to the differences in antigens used [20]. Third, it is to be expected that the true added value of anti-DFS70 detection in a routine context is also dependent on the request pattern and the setting of the laboratory (primary care versus secondary or tertiary care). Based on the idea that anti-DFS70 are more frequent in healthy and that isolated anti-DFS70 are rare in AARD, it is to be expected that in populations with higher pretest probability of AARD, the prevalence of anti-DFS70 is lower and the co-occurrence of anti-DFS70 with other AARD-associated ANA is higher, resulting in lower clinical added value. However, no formal studies have been performed to document this assumption.

The purpose of this multicenter study was to evaluate the added value of detecting anti-DFS70 in different routine contexts. Therefore, we pooled data from four Belgian laboratories in different care settings and compared the frequency and co-occurrence of anti-DFS70 with other AARD-associated ANA. Also demographics and clinical data were collected.

Materials and methods

Samples

Consecutive samples of adult patients (≥ 18 years) for which ANA-IIF was requested were included in this study. Samples originated from four Belgian clinical routine laboratories in different care settings: Algemeen Medisch Laboratorium Antwerp (AML) (primary care), OLV Hospital Aalst (OLVA) (secondary care), GZA Hospitals Antwerp (GZA) (secondary care) and University Hospital Ghent (UZG) (tertiary care). The number of samples included and the collection timeframe in each center are shown in Table 1. As the recognition of the DFS70-ANA-IIF pattern is difficult, definite inclusion for further anti-DFS70 analysis was based on the presence of a homogeneous ANA IIF pattern (i.e. homogeneous and regular fluorescence of the interphase nuclei and metaphase chromatin) or a speckled mitosis-positive ANA IIF pattern (i.e. all types of speckled fluorescence of interphase nuclei in combination with a speckled aspect of the metaphase chromatin),

Table 1: Overview of the material and methods used in the different centers.

	AML	GZA	OLVA	UZG
Numbers of consecutive samples (n)	n = 11018	n = 1481	n = 1109	Cohort 1 = 192 Cohort 2 = 430 Total: n = 622
Collection period	May 2015–April 2016	May–October 2015	May–June–September 2015	Cohort 1: End February–mid March 2013 Cohort 2: End July–end August 2016
ANA-IF method and manufacturer (serum dilution used for screening)	HEp-2 Euroimmun (1:80)	HEp-2 Inova (1:160)	HEp-2 Inova (1:80)	HEp-2000 Immunoconcepts (1:40)
Anti-DFS70 method (manufacturer)	ELISA ^a (Euroimmun) CLIA ^b (Inova)	LB (Euroimmun) CLIA (Inova)	ELISA (Euroimmun) CLIA (Inova)	ELISA (Euroimmun) CLIA (Inova)
Anti-ENA method – Screen (manufacturer)	FEIA CTD screen (ThermoFisher)	N.P.	ELISA ANA screen 11 (Euroimmun)	FEIA symphony (ThermoFisher)
– Confirmation (manufacturer)	FEIA single tests (ThermoFisher)	Euroline Profile 3 (Euroimmun)	Euroline Profile 3 (Euroimmun)	FEIA single tests (ThermoFisher) Euroline Profile 5 (Euroimmun)
Anti-dsDNA method (manufacturer)	FEIA (ThermoFisher) ELISA Quanta Lite SC (Inova) Crithidia (Euroimmun)	Crithidia (Inova)	ELISA dsDNA-NcX (Euroimmun) Crithidia (Euroimmun)	Crithidia (Immunoconcepts) Farr (Trinity)

ELISA, enzyme-linked immunosorbent assay; CLIA, chemiluminescence immunoassay; LB, lineblot; FEIA, fluorescence enzyme immunoassay; N.P., not performed; ^aperformed from May 2015–November 2015; ^bperformed from December 2015–April 2016.

further referred to as the homogeneous-like group. All serum samples were obtained as part of routine screening for ANA. No informed consent was needed for this retrospective study, but the study was performed according to the Declaration of Helsinki and approved by the Local Hospital Ethics Committee.

Routine ANA-IIF analysis and confirmatory tests

All samples were tested for ANA-IIF, double-stranded DNA antibodies (anti-dsDNA) and extractable nuclear antibodies (anti-ENA) using the routine method in each center. See Table 1 for more details on the methods used. For ANA-IIF analysis, three centers used HEP-2, and one center used HEP-2000. For the detection of anti-ENA, three centers used a screening method with pooled antigens before further identification of the specific reactivities. As the screening dilution for ANA-IIF routine analysis differed between centers, the data were aligned towards a cutoff for positivity of 1:160.

Detection of DFS70 antibodies

Three different techniques for the detection of anti-DFS70 were used in this study: one enzyme-linked immunosorbent assay (Anti-DFS70 ELISA, EUROIMMUN AG, Lübeck, Germany) (DFS70 ELISA), one lineblot (Euroline ANA Profile 3 plus DFS70, EUROIMMUN AG, Lübeck, Germany) (DFS70 LB) and one chemiluminescence immunoassay (QUANTA Flash® DFS70, Inova diagnostics, San Diego, CA, USA) (DFS70 CLIA). The DFS70 ELISA and LB methods use the full-length (FL) protein (aa 1-530), and the DFS70 CLIA method uses a truncated (TC) antigen (aa 349–435). For all assays, the manufacturers' cutoff was applied. In three centers (GZA, OLVA and UZG) DFS70 antibodies were detected using two different solid phase techniques (see Table 1). In the primary care center (AML), only one technique was used (DFS70 ELISA or CLIA depending on the timeframe). All DFS70-positive samples (positivity with at least one technique) from the secondary and tertiary care centers were also analyzed with an immunoabsorption method (NOVA Lite® HEP-2 Select, Inova diagnostics, San Diego, CA, USA).

Data analysis

All data evaluations were performed using MedCalc® Software version 15.6.1 (Ostend, Belgium). The Mann-Whitney test was used to evaluate the difference between two independent samples. For comparison of proportions, χ^2 -testing with Yates' correction for continuity was applied. A statistical significance level of 0.05 was used. In case of multiple testing, the Bonferroni correction was applied.

Results

Description of the referral pattern

The demographics of each population and characteristics of the referral pattern per center are given in Table 2. Higher numbers of referrals from rheumatologists were observed in secondary and tertiary care compared to primary care.

ANA-IIF positivity and frequency of the homogeneous-like pattern in the different cohorts

The highest ANA-IIF positivity in routine was observed in the UZG (58%) and the OLVA (52%), whereas lower frequencies were observed in AML (23%) and GZA (18%). However, it must be mentioned that UZG applies a lower screening serum dilution (1:40) compared to OLVA (1:80), AML (1:80) and GZA (1:160). At identical cutoff of 1:160, a total of 694 samples showed a homogeneous-like pattern on ANA-IIF. The frequency of this pattern within each individual consecutive cohort was 3% ($n = 327/11018$) for AML, 8% ($n = 50/622$) for UZG, 9% ($n = 138/1481$) for GZA and 19% for OLVA ($n = 211/1109$).

Differences between solid phase assays for anti-DFS70 detection

In three centers (GZA, OLVA and UZG, $n = 367$), DFS70 antibodies were detected using two different solid phase techniques (see Table 1): one technique using the TC antigen (DFS70 CLIA) and one technique using the FL antigen (DFS70 ELISA or LB). Overall, a kappa agreement of 0.749 (95% confidence interval [CI] 0.659–0.859) between the FL and TC antigen-based assays was observed. In total, 49 (13%) of 367 samples showed reactivity in at least one assay, 63% ($n = 31/49$) of them being positive in both assays. Most single positive samples ($n = 17/18$) showed reactivity in an FL antigen assay (DFS70 ELISA or LB).

Table 2: Description of the consecutive sample cohorts and characteristics of the referral pattern per center.

	AML (n = 11018)	GZA (n = 1481)	OLVA (n = 1109)	UZG (n = 622)
Median age (range)	51 year (18–101)	54 year (18–95)	53 year (18–95)	50 year (18–89)
Male/female (female%)	3479/7539 (68.4%)	530/951 (64.2%)	344/765 (69.0%)	195/427 (68.6%)
% Requests from rheumatologists	5%	26%	55%	31%

Frequency of anti-DFS70 in the different cohorts

In total, 134 of 694 homogeneous-like samples were found positive for anti-DFS70 using a combination of the solid phase techniques. See Table 3 for an overview of the positive samples in each center in relation to the different assays. When only anti-DFS70 reactivity on CLIA (55 samples in total) was taken into account to calculate the frequencies over the different cohorts, the highest frequency of anti-DFS70 within the homogeneous-like population was observed in AML (23%, $n=23/101$), with a trend towards lower frequency in tertiary care (UZG, 10%, $n=5/50$) and secondary care (GZA [13%, $n=14/106$], OLVA [6%, $n=13/211$]). Nevertheless, significance could only be shown for OLVA ($p < 0.001$).

Co-occurrence of anti-DFS70 with other ANA

Co-occurrence of anti-DFS70 with other AARD-associated ANA as determined by the routine methods was observed in 9% ($n=5$) of the 55 DFS70 CLIA-positive samples (see Table 3 for individual numbers of the centers). Higher frequencies of co-occurrence were found in the FL assay (DFS70 LB or ELISA) positive samples (31%, $n=15/48$ [only tertiary and secondary care taken into account]), with up

to 71% ($n=12/17$) co-occurrence in the positive samples without concurrent CLIA reactivity (single FL assay positivity, $p < 0.0001$).

The frequency of DFS70 CLIA reactivity without concomitant routinely detected AARD-associated ANA ranged between 6% and 21%, with a trend towards lower frequency in tertiary care (UZG 8%, $n=4/50$) and secondary care (GZA [12%, $n=13/106$], OLVA [6%, $n=12/211$]) compared to primary care (AML 21%, $n=21/101$). However, only the comparison between OLVA and AML was found to be statistically significant ($p < 0.001$).

All DFS70-positive samples originating from the secondary and tertiary care centers were also analyzed with the NOVA Lite® HEp-2 Select immunoabsorption method to determine isolated anti-DFS70 reactivity on IIF. Using this approach, 78% ($n=25/32$) of the DFS70 CLIA positive samples showed a pattern suggestive for isolated anti-DFS70 IIF reactivity. By contrast, all samples with single FL assay reactivity ($n=17$) had an ANA-IIF pattern on HEp-2 Select suggestive of the co-occurrence of other ANA reactivities, and in 64% ($n=11$) of them, the specific reactivity was also identified in the routine anti-ENA/dsDNA methods. The most frequently observed reactivity was anti-Scl70 (in 7/17 [41%]). Other reactivities were anti-Sm ($n=2$), anti-Ro52 ($n=4$), anti-Ro60 ($n=3$), anti-SSB ($n=1$) and anti-dsDNA ($n=1$).

Table 3: Numbers of samples showing the homogeneous-like pattern and DFS70 reactivity over the different cohorts and in relation to the different solid phase techniques.

Number of samples	Total	AML	GZA	OLVA	UZG
Homogeneous-like samples	694	327 ^a	106 ^b	211	50
Anti-DFS70-positive samples based on positivity with at least 1 technique [% of homogeneous-like samples]	134 [19%]	85 ^a [26%]	20 [19%]	16 [7.6%]	13 [26%]
Anti-DFS70-positive samples in relation to the applied technique					
– CLIA+	55	23	14 ^c	13 ^c	5 ^c
– FL assay+	110	62	19	16	13
– Single CLIA+	1 ^e	NA ^d	1	0	0
– Single FL assay+	17 ^e	NA ^d	6	3	8
Anti-DFS70-positive samples without concurrent ANA-reactivity in relation to the applied technique ^f					
– CLIA+	50	21	13	12	4
– FL assay+	91	58	15	13	5
– Single CLIA+	1 ^e	NA	1	0	0
– Single FL assay+	5 ^e	NA	3	1	1

CLIA, chemiluminescence immunoassay using the truncated antigen; FL assay, lineblot or enzyme-linked sorbent assay using the full-length antigen; NA, not applicable. ^aOne hundred and one samples were analyzed with CLIA of which 23 samples were positive on DFS70 CLIA; 226 samples were analyzed with ELISA of which 62 were found positive. ^bThirty-two samples were excluded because of sample shortage or double inclusion in the consecutive series. ^cAll (except 1 GZA sample) showed double reactivity for CLIA and a full-length antigen-based assay (lineblot or ELISA). ^dSamples were analyzed by either CLIA or ELISA. ^eOnly data from secondary and tertiary care centers were included. ^fDefinition of concurrent ANA reactivity based on the routine anti-ENA/dsDNA tests.

Demographic and clinical characterization of the anti-DFS70-positive samples

We evaluated the characteristics of the isolated anti-DFS70 CLIA-positive samples (comparison of isolated DFS70+ versus isolated DFS70-). The results are summarized in Table 4. A female dominance in the isolated anti-DFS70-positive cohorts was observed for three out of the four centers without significant difference. For two centers, the isolated anti-DFS70-positive patient group was significantly younger than the negative group.

No significant differences in the proportion of ANA-IIF titers $\geq 1/160$ were observed.

We also documented the association with AARD in the anti-DFS70-positive samples of three cohorts (UZG, GZA and OLVA) (Table 5) by retrospective review of the medical records and laboratory data. Considering all DFS70-positive samples (at least one method DFS70 positive), the highest association between AARD and anti-DFS70 positivity was observed in UZG (77%, $n=10/13$). A lower frequency of AARD was observed in the secondary care centers (25% [$n=5/20$, GZA] and 6% [$n=1/16$, OLVA]).

Table 4: Characteristics of the isolated anti-DFS70 CLIA-positive samples.

Number of samples	AML	GZA	OLVA	UZG
Number of isolated anti-DFS70 CLIA+ samples within the homogeneous-like cohort	21/101 ^{a,b}	13/106 ^a	12/211 ^a	4/50 ^a
Female, %				
Isolated DFS70+	76%	82%	80%	100%
Isolated DFS70-	89%	76%	75%	83%
p-Value (χ^2)	$p=0.2595$	$p=0.9373$	$p=0.9923$	$p=0.8422$
Age (median, [range])				
Isolated DFS70+	41 [25–83]	41 [21–82]	46 [20–76]	51 [28–56]
Isolated DFS70-	59 [19–91]	57 [22–88]	57 [24–91]	51 [19–86]
p-Value (Mann-Whitney) ^c	$p=0.00005$	$p=0.2612$	$p=0.0253$	$p=0.6041$
ANA-IIF titer (% with titer $\leq 1/160$)				
Isolated DFS70+	81%	54%	25%	NP
Isolated DFS70-	68%	37%	36%	NP
p-Value (χ^2)	$p=0.0924$	$p=0.2404$	$p=0.4469$	

CLIA, chemiluminescence immunoassay; NP, not performed. ^aDefinition of isolated reactivity based on the anti-dsDNA and anti-ENA routine assays. ^bFor AML, only samples performed on CLIA ($n=101$) were taken into account. ^cSignificant difference shown in bold.

Table 5: SARD association in anti-DFS70-positive samples.

	GZA	OLVZ	UZG
Anti-DFS70-positive samples (positivity with at least one technique)			
Number	20	16	13
n SARD+	5	1	10
% SARD+	25%	6%	77%
Type SARD ^a	SLE ($n=5$)	SSc ($n=1$)	SLE ($n=1$), SSc ($n=9$)
Anti-DFS70 CLIA+			
Number	14	13	5
n SARD+	1/13 ^b	0/13	3/5
% SARD+	8%	0%	60%
Type SARD ^a	SLE ($n=1$)		SLE ($n=1$), SSc ($n=2$)
Isolated anti-DFS70 CLIA+ ^b			
Number	11	10	4
n SARD+	0/10 ^c	0/10	2/4
% SARD+	0%	0%	50% SARD
Type SARD ^a			SLE (1), SSc (1)

SLE, systemic lupus erythematosus; SSc, systemic sclerosis. ^aAssociation with SARD documented based on retrospective review of the medical records and laboratory data. ^bDefinition of isolated reactivity based on the Hep-2 Select assay. ^cNo clinical data available for 1 anti-DFS70-positive patient.

Focusing on the isolated anti-DFS70 CLIA-positive samples defined using HEp-2 select analysis, the association with AARD was lower (50% in UZG, $n=2/4$) and absent in the secondary care centers.

Strategy for routine analysis

We also evaluated the possible routine impact of detecting DFS70 antibodies on every homogeneous-like sample without concomitant AARD-associated ANA. We calculated that extra anti-DFS70 testing would be necessary in 16% of the 1109 consecutive samples in OLVA (173 samples showed a homogeneous-like pattern on IIF with no AARD-associated ANA in the routine tests), in 5% for UZG ($n=30/622$), in 4% for GZA ($n=62/1481$) and in 2% for AML ($n=91/4180$, consecutive samples analyzed from December 2015 to April 2016). The chances of finding anti-DFS70 in these samples eligible for testing were 21% (GZA, $n=13/62$), 13% (UZG, $n=4/30$), 7% (OLVZA, $n=12/173$) and 23% ($n=21/91$) for AML. The frequencies of isolated anti-DFS70 within the homogeneous-like cohorts (UZG 8%, GZA 12%, OLVA 6% and AML 21% – see above) match the estimated decrease in anti-ENA/dsDNA testing based on the assumption that no testing will be performed during follow-up in case of isolated anti-DFS70 positivity.

Discussion

Today, it is accepted that screening for ANA by IIF remains indispensable in the differential diagnosis of AARD [3]. In addition, ANA-IIF analysis is, more frequently than before, ordered in a wider clinical framework, resulting in lower pretest probability of AARD. Against this background of a less focused request pattern, several authors reported the presence of anti-DFS70 antibodies (1%–15%) in patients referred for ANA testing [2, 21–26]. In our study on different consecutive populations referred for ANA testing, we detected 0.5% anti-DFS-70 using CLIA in primary care, 0.9%–1.2% in secondary care and 0.9% in tertiary care. Therefore, we cannot confirm the suggestion of Seelig and colleagues that a lower prevalence of DFS-70 antibodies is to be expected with higher quota of AARD patients within the clientele [7]. Nevertheless, we observed a trend towards lower frequency of anti-DFS70 within the homogeneous-like samples on ANA-IIF in tertiary care (10%) and secondary care (13% and 6%) compared to primary care (23%). Previous findings showing that anti-DFS70 were more frequent in younger patients were confirmed in three out of four cohorts. By contrast, significance could

not be shown for the previously reported higher frequency of females [15, 17].

The laboratory identification of anti-DFS70 has some important challenges. Indeed, differences in frequency of anti-DFS70 are observed depending on the detection technique used, and even within methods, variability is observed [17, 19, 20]. For ANA-IIF, the differences have been assigned to the subjectivity of the interpretation as well as to substrate variability [17, 19]. Moreover, some authors also suggested that in a routine context, the frequent mixed homogeneous and speckled patterns or quasi-homogeneous patterns are difficult to distinguish from the characteristic DFS70 ANA-IIF pattern [27]. In addition, it has been postulated that there are likely also other antigen targets that produce a DFS IIF pattern. One target recently suggested was MeCP2 (methyl CpG protein 2), but the presence of antibodies targeting this antigen could not be confirmed in human sera [28]. This variability/difficulty was also observed in our study with differences observed between HEp-2 and HEp-2000 results as well as interreader variability (data not shown). To overcome this, we did not use the criterion of the characteristic DFS pattern for inclusion for further testing, but we applied the broader homogeneous-like criterion. The frequency of the homogeneous-like pattern within each cohort ranged between 3% and 19% (at titer 1:160). The higher frequency of homogeneous-like samples in one of the secondary care centers compared to tertiary care is in line with the higher frequency of requests originating from the rheumatology department.

We also compared anti-DFS70 data from different solid phase assays. Our results revealed a substantial agreement between CLIA (based on TC antigen) and the FL antigen assays (DFS70 ELISA or LB, both from the same manufacturer). However, it was remarkable that the discordant samples were mostly single positive with the FL antigen assays. Our observations are in line with the findings of Bizzaro and colleagues [20], who also identified more anti-DFS70 using Euroimmun assays compared to CLIA. Theoretically, this higher sensitivity of the FL antigen assays compared to TC antigen assays, could be related to targeted epitopes not included in the TC recombinant antigen. Nevertheless, their results suggested that the differences in the assays are not reducible to differences in type of antigen used (TC vs. FL), as higher sensitivity compared to CLIA was also observed with another TC antigen assay. The same authors also suggested a possible impact of the applied cutoff for positivity. This was compatible with the results of our study, as most discordant samples showed low positivity in the FL assays (data not shown) and presence

of AARD-related ANA. In addition, we observed in an unselected consecutive cohort of routine samples (UZG, $n=186$) that discordant anti-DFS70 samples ($n=8/9$) did not show an isolated homogeneous-like pattern on ANA-IIF (data not shown). Within this context, we suggest not to report anti-DFS70 in absence of an isolated homogeneous-like pattern.

Recent observations suggest that especially anti-DFS70 in absence of other AARD-related ANA (referred to as 'isolated anti-DFS70') might contribute to explaining a significant proportion of these AARD-unrelated ANA-IIF-positive results [7, 8]. Within this context, we would also like to highlight that the definition of isolated anti-DFS70 differs between studies. We used in our study two approaches: (1) based on negative results in the routine anti-ENA/dsDNA assays and (2) based on the disappearance of the DFS-pattern on IIF after immunoabsorption. The latter approach allows for a more narrow definition of isolated anti-DFS70 as also ANA-IIF-positive reactivities not included in the classical anti-ENA assays are detectable. In our study, co-occurrence of anti-DFS70 with routinely detected AARD-associated ANA was mostly observed in the FL DFS70-positive samples (overall 31%, up to 71% in the subset with single FL antigen reactivity). In the samples with CLIA DFS70 reactivity, co-occurrence with routinely detected AARD-associated ANA was lower (7%–20%), with the highest frequency observed in tertiary care. In these samples, the highest AARD-association was also observed (see also below). Globally, these observations suggest a higher specificity of DFS70 CLIA and highlight the importance of focusing on the isolated presence of anti-DFS70.

Some authors suggested that the isolated presence of anti-DFS70 could be used as an exclusion marker for AARD, a suggestion based on the observation of lower prevalence of anti-DFS70 in AARD (median 3.9%) compared to healthy persons (7.6%) [7]. This idea was also supported by the lower median prevalence of isolated anti-DFS70 in AARD patients (<1%) [7]. Nevertheless, in healthy donors, the coincidence of anti-DFS70 with ANA, and especially AARD-associated ANA, was not always thoroughly investigated [7]. We found between 0% and 77% AARD association within our anti-DFS70-positive cohort (depending on the definition of anti-DFS70 positivity and cohort). Therefore, our data do not support that anti-DFS70 can exclude AARD. To our opinion, in a positive ANA IIF sample, it is rather the absence of a AARD-related ANA and clinical symptoms that contributes in the exclusion of AARD than the presence of anti-DFS70. Nevertheless, we can agree that anti-DFS70 may explain

positive ANA-IIF results, contributing in the clarification of diagnostic challenges.

Some authors claimed that measurements of anti-DFS70 are cost-effective in patients with AARD suspicion by comparing costs of follow-up laboratory testing and follow-up clinic visits after integration of anti-DFS70 analysis in their workup algorithm [29]. They calculated a cost saving of laboratory costs of about 17,000 euros by applying their new algorithm. This was based on the presence of anti-DFS70 in 23 samples of a total of 170 ANA-IIF homogeneous or speckled positive samples (13%). Unfortunately, details on the result calculations are not mentioned, but a decrease of 50% for anti-ENA and 75% for anti-dsDNA after implementation of their new approach is described. For comparison, we estimated a decrease of 8%–21% in anti-ENA/dsDNA tests based on the assumption that no testing will be performed during follow-up in case of isolated anti-DFS70 positivity. These differences emphasize on the importance of taking into account the care setting (or alternatively the referral pattern or origin of the requests) when performing cost-effectiveness studies.

Our study has some limitations. First, as this study followed out of the real routine laboratory situation, all centers used their established routine method for ANA-IIF and the detection of anti-ENA and anti-dsDNA. Therefore, we cannot exclude possible differences between centers in the definition of homogeneous-like samples as well as the definition of samples without AARD-associated ANA. Second, our study did not take into account possible differences between the two methods using FL antigen as both methods were produced by the same manufacturer, and only one assay with truncated antigen was performed. Third, the clinical association study was limited to the secondary and tertiary care samples.

In conclusion, the anti-DFS70 prevalence depends on the applied assays and the care setting, with anti-DFS70 CLIA positivity being most prevalent in primary care. Our data suggest that, in a patient with a positive ANA-IIF result, it is rather the absence of a AARD-associated ANA and clinical symptoms that contribute to the exclusion of AARD than the presence of anti-DFS70. Nevertheless, anti-DFS70 in absence of AARD-associated ANA may contribute to the clarification of positive ANA-IIF results, especially if pretest probability for AARD is low. Cost-effectiveness studies are useful but should take into account the care setting/referral pattern/origin of the requests.

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