Prevalence and serological profile of anti-DFS70 positive subjects from a routine ANA cohort

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Anti-Dense Fine Speckled 70 (DFS70) antibodies are a common finding in clinical laboratory referrals. High prevalence of DFS70 autoantibodies in healthy population and usual negative association with Antinuclear Antibody (ANA)-associated autoimmune rheumatic diseases (AARD) were reported. The aim of this study was to evaluate the prevalence of DFS70 autoantibodies and their association with other autoantibodies in the context of a routine ANA referral cohort. Consecutive sera submitted for ANA screening were analyzed for anti-DFS70 antibodies by indirect immunofluorescence (IIF) (n = 3175, 1030 men and 2145 women) then confirmed by immunoblotting. Anti-DFS70 positive samples were also assayed for a large spectrum of other circulating autoantibodies. The prevalence of anti-DFS70 antibodies was 1.7% in the whole population and 4.6% in the ANA-positive samples. Comparison between DFS70 IIF and immunoblotting showed an excellent correlation between the two methods. The prevalence of anti-DFS70 positive was significantly higher in females (2.1%, 45/2145) than in males (1.0%, 10/1030). Of note, no concomitant autoantibodies were found in the DFS70-positive male group compared with DFS70-positive females group that showed other serum autoantibodies in the 51% of cases. Anti-DFS70 reactivity in male population may represent an useful biomarker predicting the absence of other autoantibodies. On the contrary, the serological profile of DFS70-positive females required further investigations in order to define the presence of concomitant disease-marker autoantibodies.
Anti-DFS70 antibodies were initially described in patients with interstitial cystitis and later in heterogeneous chronic inflammatory conditions, tumors and even in apparently healthy individuals, but their clinical impact is still unknown. It was reported that none of anti-DFS70 positive subjects showed symptoms suggestive of an AARD after clinical follow-up of 4 years.

Some previous studies suggested that the isolated anti-DFS70 reactivity could be taken as biomarker to exclude AARD (Antinuclear Antibody (ANA)-associated autoimmune rheumatic diseases) from ANA-positive healthy individuals. Understanding the serological and clinical profile of anti-DFS70 positive subjects thus could avoid inappropriate referral to care specialists and follow-up tests for healthy people. Accordingly, the aim of this study was to define the prevalence of anti-DFS70 antibodies in a routine diagnostic laboratory setting and the associated serum autoantibodies to support the clinical use of these markers.

Methods

Samples. Collected samples consisted of serum specimens sent to the Immunopathology Laboratory of the San Carlo Hospital of Potenza by outpatients or inpatients (Cardiology, Dermatology, Endocrinology, Geriatrics, Gynaecology, Haematology, Infectious Disease, Internal Medicine, Nephrology, Neurology, Oncology, Ophthalmology, Pneumology and Rheumatology Units) for ANA analysis with IIF. Paediatric subjects were excluded. Confirmed IIF DFS70 positive adult patients were asked to participate in the present study. Recruited patients have given their informed consent. Collection of patient samples and laboratory procedures were carried out according to Common Regional Ethical Committee of Basilicata (CEUR) (Authorization Number: 705/2017).

ANA Testing. ANA were detected by commercial ANA HEP-2000 Indirect Immunofluorescent assays (HEP-2000 Fluorescent ANA-Ro Test System, Immuno Concepts N.A., Sacramento, CA, USA). ANA Kits were used according to the appropriate manufacturer’s instructions. The screening dilution was 1:160. Automated instrument for IIF preparation (Gemini Combo, Stratec Biomedical) and anti-human IgG specific fluorescein-labelled (FITC) conjugate were used. Subsequently, slides were assessed with a fluorescence microscope. For each well, 4 microscopic fields with 20x objective with Image Navigator (Immuno Concepts N.A., Sacramento, CA, USA) were captured. This instrument is based on a conventional fluorescence microscope with a motorized stage, high-intensity LED light source, digital camera, computer and proprietary software. Manufacturer’s Fluorescence Index (FI) cut-off value for HEP-2000 is equal to 30. Positive/negative interpretation based on a FI was evaluated during focusing and confirmed by expert-readers. Positive samples were also tested using the conventional serial dilution method (1:160, 1:320, 1:640, 1:1280 until 1:2560) to assign end-point titer. The titer was defined as the reciprocal of the highest dilution of serum that still shows immunofluorescent staining.

Immunoblot Anti-DFS70 Assay. The serum samples positive for DFS70-like pattern in IIF were further processed for detection of human IgG autoantibodies against DFS70 specificity (truncated sequence of the DFS70 antigen (residues 349–435)) by Immunoblotting assay. In addition, confirmed anti-DFS70 positive samples, were also evaluated for the antibodies against Sm, U1-RNP, Sm/RNP, SSA/Ro60kD, SSb, Scl-70, PM-Scl 100, Ku, CENP-A/B, PCNA, Mi-2 antigens using immunoblotting kit (Alaphidia, Wavre, Belgium) by automatic Blu Diver Instrument (BDI) (Alifax, Polverara (PD), Italy). During the automated test procedure, the BDI sequentially incubates the strips in the wells of ready-to use reagent cartridges. Human antibodies bind the corresponding specific antigen(s) on the membrane and enzyme activity leads to development of purple dots on the membrane pads. The intensity of the coloration is directly proportional to the amount of antibody present in the sample. Semi-quantitative interpretation was done by Dr Dot Software (Alifax) and scanning system using Arbitrary Unit (AU).

Detection of other autoantibodies. Anti-DFS70 positive samples were tested for the presence of other serum autoantibodies: anti-extractable nuclear antigen (ENA) (RNP/Sm, SSA/Ro, SSB/La, Scl-70, Jo-1, CENP-B), anti-thyroid peroxidase (TPO), anti-thyroglobulin (TG) and anti-tissue transglutaminase (TGt)-IgA by a fully automated chemiluminescence analyzer LIAISON XL (DiaSorin, Saluggia (VC), Italy) using a ‘Flash’ chemiluminescence technology (CLIA) with paramagnetic microparticle solid phase; anti-dsDNA by the Crithidia luciliae IIF Test (Euroimmun, Padova (PD), Italy); IgG class of anti-mitochondrial (AMA), anti-smooth muscle (ASMA), anti-liver/kidney/microsome type 1 (LKM), and anti-parietal cells (PCA) by triple IIF test on rat liver, stomach and kidney substrates (Alphaphia); anti-neutrophil cytoplasmic myeloperoxidase (MPO) and proteinase 3 (PR3) through enzyme immunoassay (GA GENERIC assays GmbH, Dahlewitz, Germany) performed on automatic Immunomat Serion ELISA analyzer (SERION Immunologics, Wurzburg, Germany) as recommended by the manufacturer; anti-Saccharomyces Cerevisiae IgG/IgA (ASCAs) and anti-cardiolipin (β2-GPI complex and β2-GPI isolated protein IgG (aCL) using immunodot kit, a previously described diagnostic assay platform.

Statistical analysis. Statistical analyses were performed using the Graph Pad Prism statistical package (v5; Graph Pad Software, San Diego, CA). Statistical significance was calculated using the non-parametric Mann-Whitney-U test and correlations were analyzed by the Spearman’s rank correlation test and R-squared analyses (R²); categorical variables were compared using Fisher’ Test. A p value < 0.05 was considered statistically significant.

Ethics approval and consent to participate. The study was approved by the Regional Ethics Committee (Authorization Number: 705/2017).
Prevalence of DFS70 antibodies. Selected cohort included 3175 sera (M = 1030, F = 2145) from consecutive adult subjects submitted for routine ANA testing. Only 45.8% (55/120) of the presumptive DFS70 pattern on the ANA-IIF test were confirmed by immunoblotting, therefore sera negative by dot blot method were included in ANA positive group. Anti-DFS70 antibodies (Fig. 1) were observed in 1.7% (55/3175) of whole series and 4.6% (55/1191) in the ANA-positive samples. The prevalence of anti-DFS70 positive females (2.1%, 45/2145) was statistically significant higher than males (1.0%, 10/1030) (p = 0.02). We found a striking female predominance both in ANA positive group (F/M, 903/288) that in anti-DFS70 positive samples (F/M, 45/10). Comparison among the mean ages of the ANA negative series (51.6 ± 16.8 yrs) versus anti-DFS70 negative/ANA positive group (52.6 ± 17.1 yrs) and anti-DFS70 positive subjects (49.5 ± 15.5 yrs) showed no statistically significant differences (p > 0.05). No statistical difference could be shown between the age in both sexes.

Analysis of the Referring Physician pattern of samples displaying Dense Fine Speckled pattern. Analyzed samples were obtained by outpatients and inpatients coming from various Departments of the San Carlo Hospital of Potenza. About anti-DFS70 positive subjects, 5.3% came from Internal Medicine, 3.6% from Neurology, Haematology and Rheumatology, respectively, 1.8% from Cardiology, 1.8% from Endocrinology and remaining 80.3% from outpatients. By comparative analysis of referring clinical departments emerged that anti-DFS70 autoantibodies were more prevalent in samples from outpatients (2.1%, 44/2096) versus inpatients (1.2%, 9/760) and Rheumatology (0.6%, 2/319) (Fig. 2).

Quantitative comparison of anti-DFS70 antibodies IIF titers and immunoblotting. Using IIF on HEp-2000 substrate at screening dilution of 1:160, 55 anti-DFS70 positive samples were identified as showing...
the typical DFS70 staining pattern. Titer distribution revealed no statistically differences when compared with other ANA patterns (p > 0.05). More than half of the patients presenting DFS70 monopattern (63% of the total cohort) showed high titers (≥1:640) (Fig. 3). A quantitative comparison between DFS70 IIF titers and AU immunoblotting showed an excellent correlation between two methods as expressed by a Spearman correlation (Fig. 4A). Regression analysis (R² = 0.99) also confirmed the relationship between IIF titers and immunoblotting AU (Fig. 4B).

**Association of anti-DFS70 and other serum autoantibodies.** Collected DFS70-positive serum samples were assayed for a large spectrum of other circulating autoantibodies. By comparing DFS70-positive males with females, it emerged that isolated anti-DFS70 reactivity was found only in the male group. In contrast, in the 51% of DFS70-positive females, concomitant serum autoantibodies were found. Most common detected autoantibodies were anti-TPO (16.0%), anti-TG (11.0%), anti-tTG-IgA (9.0%) and ANCA (2.2% anti-PR3, 2.2% anti-MPO and 2.2% both). Only two subjects had both anti-TPO and anti-TG antibodies. ASCAs were detected only in one DFS70-positive female, also positive for anti-TPO, anti-TG and anti-tTG-IgA; anti-cardiolipin in two other and anti-ENA (anti-Mi-2 antibody) in one which also showed anti-TPO antibody positivity. No anti-DFS70 positive female, carried concomitant anti-dsDNA, anti-PCA, AMA, ASMA and anti-LKM was found (Fig. 5).

**Clinical phenotypes of anti-DFS70 positive patients.** Clinical features of anti-DFS70 positive subjects were as follows: 37 healthy subjects (29 F, 8 M) and remaining (16 F, 2 M) suffered from various diseases. In the latter group 3 had spondyloarthritis (SpA), 1 morphea, 1 undifferentiated connective disease (UCTD), 3 rheumatoid arthritis (RA), 1 alopecia areata, 1 Crohn's disease, 4 celiac disease (CD), 1 autoimmune thyroiditis (AT), 1 RA-UCTD overlap syndrome, 1 CD plus AT, and 1 SpA plus AT.
Discussion

Previous studies using ANA-IIF test on HEp-2 substrates provide the detection of high-titer autoantibodies with typical DFS70 staining pattern in 1% to 16% of the studied cohorts. These heterogeneous results were obtained in different patient cohorts, including blood donors, healthy individuals, routine ANA cohorts, selected ANA positive healthy individuals and routine ANA positive subjects9–22. In details, a rate of positivity ranged from 0 to 5% was found in blood donors, healthy children and in routine ANA screening cohort. Higher frequencies were reported in ANA-positive populations. In addition, some studies examined serum samples only by IIF without specific confirmatory assays, resulting in higher rate of positivity. It has been showed that not all samples displaying DFS70 IIF pattern were then confirmed by specific CLIA or ELISA methods23,32,33. We analyzed 3175 consecutive unselected samples screened for ANA during the routine work-up by IIF at screening dilution of 1:160, then confirmed by immunoblotting (Fig. 1). We found 1.7% of anti-DFS70 positive sera, in line with some previous studies conducted on routine ANA screening population 6,10,13,16,19,21,34. In fact, Mahler et al.6 analyzed 3263 sera submitted for ANA screening for the presence of anti-DFS70 antibodies and observed 1.62% of positivity. Noteworthy, Bizzaro et al.15 also reported a low frequency (0.8%) of sera displaying the anti-DFS70 reactivity on HEp-2 substrate in a large cohort of samples screened for ANA in clinical laboratory. However, because our study was performed with ANA-requested specimens from outpatients and inpatients, the overall frequency of anti-DFS70 antibodies in general population is unknown. Gender differences with greater prevalence of female (4:1) was observed in anti-DFS70 negative/ANA positive group and within the anti-DFS70 positive samples. This is an important finding since the increased immune reactivity in females predisposes them to developing AARD. A number of factors may underlie this striking gender difference and further studies are needed to better understand these findings.

Regarding referring sources of anti-DFS70 positive samples, a comparative analysis highlighted that greater prevalence of anti-DFS70 positive subjects referred from the outpatients, with value of 2.1% (Fig. 2). We found high titers (≥1:640) in 64.3% of the DFS70 IIF-positive sera, with a titer greater than 1:2560 found only in one sample. Titer distribution revealed no statistically differences when compared with other ANA positivity (p > 0.05) (Fig. 3).

The use of specific methods to confirm DFS70 IIF suspicion was highly recommended. Recent data showed that there were no differences in diagnostic accuracy among methods using truncated (as Alphadia Dot) or full-length DFS70 antigenic sequence34,35. We perform a quantitative comparison among IIF titers and immunoblotting AU in anti-DFS70 positive sera resulting in excellent correlation (Fig. 4A,B).

We also analyzed the simultaneous presence of a broad spectrum of other circulating disease-markers autoantibodies. Muro et al., found a high prevalence of disease-related autoantibodies in anti-DFS70 positive patients, but of note, their cohort included only 22 anti-DFS70 positive females. Noteworthy, our data evidenced isolated reactivity of anti-DFS70 autoantibodies in male group, and high prevalence of disease-marker autoantibodies in females (51%). In particular, most common detected autoantibodies were anti-TPO, anti-TG, anti-ITg-IgA and ANCA (Fig. 5). It is hoped that future studies will compare the prevalence of concomitant antibodies in males versus females either in ANA positive/anti-DFS70 negative group.

Major strengths of our study are the use of anti-DFS70 confirmatory assay to avoid false positive results and the gender analysis in appraisal of the anti-DFS70 antibodies prevalence. The prevalence of anti-DFS70 was
previously assessed in different cohorts mostly smaller than ours, therefore, we believe that our anti-DFS70 positive cohort should be considered suitable for a preliminary evaluation although a future larger confirmatory study is needed. In contrast, the scarce number of anti-DFS70 positive males could be considered inadequate to draw any firm conclusions. Therefore, based on low male prevalence, it is mandatory to screen larger cohort by future multicentre studies.

Conclusion

According to previous observations performed on routine ANA cohorts, our prevalence of anti-DFS70 positivity was 1.7% of adult screened population. Serological profile of anti-DFS70 positive females required further clinical and laboratory investigations in order to define the presence of associated disease-marker autoantibodies and their clinical significance. In contrast, isolated anti-DFS70 specificity in male population suggests that the DFS70 could be considered reliable screening indicator for absence of other circulating autoantibodies resulting in considerable cost-saving potential.

Data Availability

The dataset generated and analyzed during the present study are not publicly available, owing to restrictions by our organization, but they are available from the corresponding author on reasonable request.

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Author Contributions
T.C., V.P. and S.D.A. designed the study, drafted the manuscript and revised it critically for important intellectual content; G.T., M.G., M.C.P. and A.A.P. made contribution to collection of data and were involved in revising the manuscript critically. All authors agreed to be accountable for all aspects of the work in ensuring that question related to the accuracy or integrity of any part of the work are appropriately investigated and resolved and approved the final version of the manuscript.

Additional Information
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