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Combining multi-antigenic immunodot with indirect immunofluorescence on HEp-2 cells improves the diagnosis of Systemic sclerosis

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Abstract

Systemic sclerosis (SSc) is associated, in nearly all patients, with autoantibodies (Ab). Accordingly, and in order to identify major (anti-CEN A/B and anti-Topo I) but also minor Abs, the usefulness of combining indirect immunofluorescence (IIF) on HEp-2 cells with an 11 multi-antigenic SSc immunodot was explored. 1689 samples tested at the request of clinicians, were evaluated retrospectively. The positivity rate was 28.8% and the diagnosis of SSc was supported for 232 samples. Two groups of Abs were considered: group 1, Abs (anti-CENP A/B, anti-Topo I) present at elevated levels in SSc patients; group 2, Abs for which the Ab specificity (odds ratio and/or positive predictive value) was improved by using IIF on HEp-2 cells (RNA-Polymerase III, fibrillarin, Th/T0, PM-Scl). Altogether, this study highlights the utility of combining IIF on HEp-2 cells with the SSc immunodot as the first line of an SSc Abs detection/SSc diagnostic strategy.

Key words: Systemic sclerosis, immunodot, HEp-2 cells, Luminex®

1. Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterized by vascular damage and fibrosis of the skin and viscera, resulting in organ failure and irreversible injury [1]. SSc is heterogeneous and can be subdivided according to the clinical features, but also by the autoantibody (Ab) profiles [2–5], the latter one having a particular interest at very early SSc stage, in comforting clinical suspicion, in borderlines forms, in rough forms or in clinical trials. SSc antinuclear Abs (ANA) have the particularities of being present in almost all patients, mutually exclusive, and directed against intracellular nuclear and/or nucleolar components present in HEp-2 cells. Among them, anti-centromere (CENP A and/or B) and anti-Th/T0 ribonucleoprotein Abs are associated with a limited disease while others (anti-Topoisomerase I [Topo I], anti-RNA Polymerase III [RNA Pol3], anti-fibrillarin/U3 RNP, and anti-U1 RNP) are associated with a poor diagnosis, predominant damage to certain organs or other autoimmune disease associations [1,6–11].

Thus, given the high sensitivity but low specificity for SSc of the IIF detection on HEp-2 cells, and given the diversity of SSc Abs, the interest of a specific and multi-antigenic assay in the diagnostic strategy has emerged. To this end we have selected a semi-quantitative multi-antigenic SSc immunodot (D-Tek) and evaluated its performance when confronted with IIF on HEp-2 cells on samples presenting at least one positive reactivity on immunodot out of the 1689 samples initially tested. Serum results confirm the potential benefits of combining SSc immunodot with IIF in HEp-2 cells as a primary approach for a quick and specific SSc Ab detection and for improving the specificity for SSc diagnostic.

2. Methods

2.1 Sample inclusion criteria

All samples tested with the multi-antigenic SSc immunodot and coupled with IIF detection on HEp-2 cells at the CHU of Toulouse between July 2016 and July 2020 were included in this retrospective study (**Figure 1**). They correspond to the Abs requested by clinicians in conjunction with clinical suspicion of SSc. Duplicates, follow up samples, and negative samples were excluded. As presented in Figure 1, SSc immunodot positive patients who fulfilled the ACR/EULAR 2013 classification criteria for SSc [12], involved

or not in an overlap syndrome, were referred to as SSc (n=232, sex ratio F/M 4.4, 57.3 years). The main clinical characteristics of SSc patients are shown in Table 1. Non-SSc patients were further subdivided according to the report by the referring doctor of an autoimmune disease (AD: n=91, sex ratio 5.1, 52.7 years) or not (Other disease, n=48, sex ratio 2.7, 55.8 years). The AD group included 25 systemic lupus erythematosus [SLE], 13 myositis and anti-synthetase syndrome, 8 Undifferentiated Connective Tissue Diseases [UCTD], 22 Sjögren's syndrome [SjS], 7 rheumatoid arthritis [RA], 5 overlap syndrome without SSc, and 3 dysimmune syndrome, 3 autoimmune thyroiditis, 2 autoimmune cirrhosis and hepatitis, 1 type 1 diabetes, 1 antiphospholipid syndrome and 1 psoriasis patient(s). In the Other group, 4 arterial hypertension, 3 cancer, 5 nephropathy, 6 various lung damage, 4 central or peripheral neurological diseases, 2 with dermatologic lesions, 3 Raynaud syndrome, 1 Behçet, 1 Horton disease, 1 HIV, 1 tuberculosis, 1 sickle cell anemia and 16 with various altered general states such as asthma, asthenia or arthralgia were included among the 48 patients presenting another diagnosis. This study has received the authorization number DC20162804 from the French ethical Southwest and Overseas committee (SOOM 2).

2.2 SSc immunodot

The multi-antigenic SSc immunodot from D-Tek company (Mons, Belgium) that allows the semi-quantitative detection of 11 antigenic targets (CEN A, CEN B, Topo I, RNA Pol3, fibrillarin/U3 RNP, U1 RNP, Ku, PM-Scl 75, PM-Scl 100, Th/T0 and Trim 21/Ro52) was selected. For Trim21/Ro52, as this parameter was included in the SSc immunodot during the study (2019), results from the D-Tek SSc or myositis immunodot were used when available (comparable results, data not shown). According to the manufacturer's instructions, sera were diluted 1:100 and immunodot strips were processed semi-automatically with the BlueDiver Instrument (D-Tek). At the end of the process, a scanner strip reading system (Dr Dot, D-Tek) allowed a semi-quantitative estimation by comparing the intensity of the spot with a grey scale included in the scanner. In each strip a positive internal control and a threshold spot are added that allows strip validation. A value greater or equal to 5 arbitrary units (AU) was considered positive.

2.3 HEp-2 cells and multiplex immunofluorescence assay

Samples were tested for ANA by IIF in HEp-2 cells (human epithelial type 2 cells, Bio-Rad) at a starting dilution of 1:160. An ANA-IIF interpretation test was done

independently by two operators and classified according to the 2016 and 2018 updated International Consensus on ANA Patterns (ICAP) [13,14]. HEp-2 cell patterns included homogeneous staining (AC-1) defined as homogeneous and regular nuclear fluorescence across all nucleoplasm and a chromatin mass intensely stained in mitotic cells; centromere staining (AC-3) defined in interphase cells as coarse speckles and in mitotic cells by an alignment at the chromatin mass; nuclear fine speckled pattern (AC-4) defined as fine tiny speckles across the nucleoplasm; large speckled pattern (AC-5) defined as coarse speckles across all nucleoplasm while the chromatin mass is not stained; and Topo I-like pattern (AC-29) considered when at interphase the nuclei is stained with a fine speckled pattern and a strong staining of the nucleolar organizing regions, and in mitotic cells when the chromatin mass is stained. For the nucleolar staining even if clumpy, homogeneous, or punctate aspects were reported, they were all grouped under the nucleolar staining term for the statistical analysis (AC-8-10).

Based on Luminex® technology, the multiplex immunofluorescence assay (Bio-Plex, Biorad, Hercules, CA) was used to detect anti-extractable nuclear antigen (ENA) Abs and among them the two SSc specific Abs, anti-CEN B and anti-Topo I Abs, were analyzed and the positive cut-off was fixed at 0.9 antibody index (AI, value range 0-8) as recommended by the manufacturer.

2.4 Statistics

Categorical data were analyzed using the Chi² test, when necessary, Yates' correction was used, and the Odds ratio (OR), positive predictive values (PPV), and negative predictive values (NPV) were calculated when appropriate. For descriptive data among groups differences were analyzed by performing a multiple Kruskal-Wallis non-parametric test and the Dunn's test was used for post-hoc comparisons. Receiver operating curves (ROC) were generated to determine the area under the curve (AUC). The percent of agreement between parameters was calculated by determining the Cohen's kappa coefficient: almost perfect (K >0.9), strong (K: 0.8-0.9), moderate (K: 0.6-0.79), weak (K: 0.4-0.59), minimal (K: 0.21-0.39), none to slight (K: 0.2 to -0.3), and disagreement (K<-0.3). P values under 0.05 were considered positive. Data were analyzed using GraphPad Prism 9.0 software (La Jolla, CA).

3. Results

3.1 Qualitative and quantitative analysis of multi-antigenic immunodot results

From July 2016 to July 2020, 1689 samples were tested at the CHU of Toulouse using the multi-antigenic SSc immunodot allowing the detection of 11 related autoantibodies (**Figure 1**). Among them, 28.8% presented a positive SSc immunodot after duplicates and follow-up samples exclusion (n=226). When considering positive samples, clinical information was available at the time of inclusion for 371 patients corresponding to 232 (62.5%) SSc according to ACR/EULAR 2013 criteria, 91 (24.5%) AD and 48 (12.9%) patients that presented another diagnosis (detailed in Method section).

Based on the cut-off fixed by the manufacturer (≥ 5 arbitrary units [UA]), two groups of reactivity can be defined according to their capacity to discriminate SSc patients from other AD and other diseases (**Figure 2A**). The first group, major SSc Abs, corresponds to Abs associated with SSc as observed for anti-CEN A ($p < 10^{-4}$), anti-CEN B ($p < 10^{-4}$) and anti-Topo I ($p < 10^{-4}$) Abs. Within group 1, anti-Topo I Abs were observed more frequently in SSc patients with a diffuse cutaneous presentation ($p < 10^{-4}$), were associated with interstitial lung disease ($p < 10^{-4}$), and SSc patients presented less telangiectasia ($p = 0.007$) as compared to SSc patients with anti-CEN A+B Abs (Table 1). As described in the literature, anti-CEN A, anti-CEN B and anti-Topo I were significantly associated with Raynaud's phenomenon [15] with an odd ratio of 9.3, 4.3 and 3.3 respectively (p -value $< 10^{-4}$), and in a less manner with esophageal dysfunction (**supplemental figure 1**). Interstitial lung disease was associated with anti-Topo I but not with anti-CEN A and B, as found in the cohorts of Jung et al. 2018 and Tangri et al. 2013 [16,17]. Within the second group of Abs, minor Ab, no significant associations with SSc or specific symptoms were observed. Among them, two predominated in the AD subgroup: anti-U1 RNP Ab ($p = 0.007$, predominant in MCTD and SLE), and anti-Trim21 Ab ($p < 10^{-4}$, predominant in SjS but also in SLE), while anti-PM-Scl 75 Ab detection ($p < 10^{-4}$) was increased in the other disease subgroup (**Figure 2A**). Considering the small sample number in the second group of Abs, sociodemographic and clinical characteristics among SSc patients were mostly similar between the various Ab from group 2 (**Table 1**).

Since the use of a scanner allowed Ab semi-quantification, values were compared between SSc, AD and other disease subgroups. Accordingly (**Figure 2B**), major SSc

associated Abs (anti-CEN A, anti-CEN B and anti-Topo I Abs) were retrieved at high levels in the SSc subgroup as compared to the AD and other diseases subgroups ($p < 0.003$). Inside the minor group of SSc Abs, for anti-RNA Pol3 Abs, higher levels were present in the SSc subgroup as compared to the AD subgroup only ($p = 0.04$) and for anti-PM-Scl 75 Abs between the SSc subgroup and Other subgroup only (**Figure 2C**). No differences were observed for the other Abs except for anti-U1-RNP, and anti-Trim21 Abs which were retrieved at significant levels in the AD subgroup ($p < 0.02$).

3.2 Anti-CEN and anti-Topo I autoantibodies

Next, SSc immunodot performance for anti-CEN A/B, and anti-Topo I Abs were compared to their IIF pattern (AC-3 and AC-29, respectively) reported in HEp-2 cells using the percent of agreement between both assays (Cohen's kappa coefficient). As presented in **Figure 3A-B**, agreement between anti-CEN A and anti-CEN B Abs using SSc immunodot *versus* the anti-CEN (AC-3) pattern in HEp-2 cells was near perfect in the SSc subgroup (kappa: 0.91 and 0.97, respectively) as compared to a moderate/strong agreement in the AD subgroup (kappa: 0.78 and 0.82, respectively) and a weak/moderate agreement in the Other diseases subgroup (kappa: 0.47 and 0.77, respectively). For anti-Topo I Abs tested by SSc immunodot (**Figure 3C**), agreement in the SSc subgroup was moderate with the HEp-2 cell AC-29 pattern ($K = 0.62$), while no agreement was reported in the AD and Other subgroups. Associations between Abs tested by SSc immunodot was evaluated showing (i) a strong concordance between the two main centromeric antigenic targets CEN A and CEN B among SSc patients ($K = 0.91$ in SSc *versus* 0.59 in ADs and $K = 0.32$ in other diseases); (ii) disagreement (defined when $k < -0.3$) between the two major anti-CEN A/B and anti-Topo I Abs in SSc individuals ($k = -0.37$) with only 2/232 (0.9%) SSc patients that possess anti-CEN A/B and anti-Topo I Abs. Another disagreement was observed in the AD subgroup between anti-Trim21 and anti-CEN B Abs ($K = -0.32$); and (iii) none or only a slight link between the other minor Abs.

Among extractable nuclear antibodies (ENA) routinely tested when ANA are positive in HEp-2 cells, anti-CEN B and anti-Topo I Abs are specific for SSc allowing their qualitative (Cohen's kappa comparison) and quantitative (ROC curves) comparison between SSc immunodot and the Luminex® immunofluorescence assay (Bio-Plex, Bio-Rad) in our study. First, regarding anti-CEN B Abs tested by SSc immunodot *versus* anti-CEN B Abs

tested by Luminex®, a near perfect agreement was observed in all subgroups (**Figure 3B**, $K>0.93$). For anti-Topo I Abs tested by SSc immunodot (**Figure 3C**), agreement was near perfect with anti-Topo I determination on Luminex® in the SSc subgroup only ($K=0.94$). Second, and to compare quantitative values obtained by SSc immunodot with those obtained by Luminex® assays, areas under ROC curves were performed between the SSc as compared to the AD and Other subgroups. As shown in **Figure 3D-F**, ROC analysis highlighted similar performances for anti-CEN A, anti-CEN B and anti-Topo I between the two technologies to discriminate SSc patients from AD and other diseases. Altogether, this supports the possibility that SSc immunodot can substitute for ENA determination in the context of SSc diagnosis for CEN B and Topo I Abs determination.

3.3 HEp-2 cell patterns for other positive SSc Abs on immunodot

As 227 (97.8%) of the 232 SSc patients have a positive IIF on HEp-2 cells, we wanted to assess whether or not coupling SSc immunodot with patterns and titrations in HEp-2 cells improves the non-anti-CEN and anti-Topo I Abs detection performance, and if it could help to discriminate the true SSc patients from others. Indeed, the cellular localization of the targets of the other SSc Abs and the appearance in HEp-2 cells is known [18], except for Trim21 which is not associated with a specific pattern in HEp-2 cells and thus removed from further analysis [19].

Regarding the two nuclear exclusive Abs (anti-RNA Pol3 and anti-U1 RNP Abs) reported in **Figure 4A/B**, among positive samples, anti-RNA Pol3 Abs were associated with a fine speckled nuclear pattern (AC-4, 95%) at elevated titer ($\geq 1/1280$, 78%) in SSc, while such performance was moderate in the AD subgroup (AC-4, 50%; titer $\geq 1/1280$, 50%) and weak in the Other subgroup (AC-4, 50%; titer $\geq 1/1280$, 25%). Anti-U1-RNP Abs were associated with a large speckled nuclear pattern in SSc and AD subgroup (AC-5, 86% and 89%, respectively, and at elevated titer ≥ 1280) but not in the Other subgroup in which no nuclear fluorescence was associated.

As presented in **Figure 4C/D**, a predominant nucleolar staining (AC-8-10, 83%) at elevated titer ($\geq 1/1280$, 83%) characterizes anti-fibrillarin Abs in SSc patients as compared to the AD and other individuals (AC-8-10 and titer $>1/1280$, $\leq 25\%$ for both).

Among the 4 latest antigenic targets (Ku, PM-Scl 75, PM-Scl 100 and Th/T0) presented in **Figure 5 A/B**, coupling SSc immunodot with IIF in HEp-2 cells was informative when

comparing the SSc subgroup (and AD subgroup) with the Other subgroup based on nucleolar staining and titer (high association in SSc and AD but not in Others).

Altogether, this suggests SSc diagnosis can be improved by associating SSc immunodot with IIF in HEp-2 cells.

3.4 SSc Immunodot performance when coupled with IIF in HEp-2 cells

Finally (**Supplementary figure 1**), SSc immunodot performance was evaluated when used alone or coupled with IIF in HEp-2 cells to first evaluate their capacity to discriminate in the cohort the major clinical phenotypes associated with SSc : Raynaud's phenomenon, interstitial lung disease, and esophageal dysfunctions. Anti-CEN A, anti-CEN B and anti-Topo I Abs were significantly associated with Raynaud's phenomenon ($3.3 < \text{Odds ratio [OR]} < 9.3$, $p\text{-value} < 0.003$), as previously described (Park et al. 2015), and with esophageal dysfunctions ($1.4 < \text{OR} < 2.4$, $p < 0.02$). Regarding interstitial lung disease anti-CEN A and/or anti-CEN B Ab detection was protective ($0.25 < \text{OR} < 0.27$, $p < 10^{-4}$), while a positive association was retrieved with anti-Topo I Abs in agreement with the literature [16,17]. Coupling immunodot with IIF in HEp-2 cells at best improved, otherwise did not influence, the characteristics of the immunodot when considering major (group 1) and minor (group 2) antibodies.

Second when comparing immunodot used alone or coupled with HEp-2 cells to discriminate SSc individuals from the AD and Other groups: OR, positive predictive values (PPV) and negative predictive values (NPV) revealed: (i) good performances for anti-CEN A, CEN B and Topo I Ab (OR =10.7 [IC95%: 5.3-21.3], 5.0 [IC95%: 3.0-8.4] , and 5.3 [IC95%:2.3-12.1], PPV= 91, 83 and 98%, and NPV=50, 50 and 42%, respectively) detection with immunodot alone and with similar performances when immunodot was coupled with the defined pattern in HEp-2 cells; (ii) an elevated performance for CEN A (OR =10.7 [IC95%:5.3-21.3], PPV= 91%, and NPV=50%) and the association with anti-CEN A/B (OR =11.9 [IC95%: 5.8-24.6], PPV= 92%, and NPV=51%) as compared to anti-CEN-B alone (OR = 5.0 [IC95%: 3.0-8.4], PPV= 83%, and NPV=50%); (iii) a capacity to discriminate SSc individuals when anti-RNA Pol3 and anti-Fib Abs are coupled with IIF in HEp-2 cells in terms of OR ($p < 0.05$ for both) and PPV (94% and 88%, respectively); (iv) a gain in predicting SSc (PPV) when immunodot is coupled with IIF in HEp-2 cells for anti-Th/T0, anti-PM-Scl 75, and anti-PM-Scl 100; and (v) no gain was reported for anti-Ku and anti-U1 RNP.

4. Discussion

In this study, we have explored the value of combining IIF detection in HEp-2 cells with the multi-antigenic SSc immunodot from D-Tek in SSc diagnosis in a retrospective French cohort. We first confirmed the predominant detection of anti-CEN A/B Abs (49.0%) and anti-Topo I Abs (21.1%) in SSc. It can be noted that despite the usefulness and robustness of tests used for ENA determination and differential diagnosis, the low number of SSc Abs (anti-CEN B and anti-Topo I) present in the ENA Luminex® based assay supports use of SSc immunodot as a first choice in the diagnosis of SSc, an assertion that is further reinforced by the similar qualitative and quantitative performances retrieved between both assays. Secondly, we have shown that associating the SSc immunodot with the IIF pattern and titers in HEp-2 cells improves SSc specificity for anti-RNA Pol3 Abs and anti-fibrillarin Abs. For the remaining SSc patients with a positive SSc immunodot (35/232, 15.1%) rarer Abs are detected that need to be coupled with IIF in HEp-2 cells for validation and with clinical characteristics since these Abs may also be reported in patients with other connective tissue diseases.

The three main characteristics of anti-CEN Abs in SSc are related to the recognition of a macro-complex, an elevated titer, and possible mutual exclusive detection with regards to anti-Topo I Abs [20]. First, interaction of CEN A (18kDa) with CEN B (80 kDa) and CEN C (140 kDa) is involved in *de novo* centromere assembly kinetochore macro-complex during mitosis explaining that circulating anti-CEN A/B titers are correlated and mutually associated with SSc, but not cross-reactive [21]. Such an association between anti-CEN A and anti-CEN B Abs was retrieved in our study within the SSc subgroup and, except for one patient with SjS (AD subgroup), all patients harboring anti-CEN A Abs were also positive for anti-CEN B Abs. Such an association is less restrictive for anti-CEN B Ab positive patients with a minor group targeting CEN B but not CEN A that was retrieved in the three clinical subgroups (Other > AD > SSc).

As a consequence, we report a better capacity to discriminate SSc when considering the association CEN A/B (OR=11.9) as compared to CEN B when tested alone (OR=5.0). Second, higher titers of anti-CEN Abs in SSc patients are reported, which may improve their detection. We reported a near perfect agreement for anti-CEN A/B Abs when detected by SSc immunodot as compared to the anti-CEN (AC-3) pattern in HEp-2 cells and to anti-CEN B Abs tested by Luminex® based-assay. Third, anti-CEN A/B and anti-

Topo I Abs are described as being mutually exclusive and when reported, double positive patients correlated with diffuse SSc [22]. This is observed in our cohort as the concomitant detection of both Abs was retrieved for two diffuse SSc cases corresponding to 0.9% of the SSc cohort. Based on a negative Kappa's concordance assay ($k < -0.3$), the negative associations between anti-CEN A or anti-CEN B Abs and anti-Trim21 Abs in the AD subgroup needs to be further explored and this is in conflict with the occurrence reported between anti-Trim21 and anti-CEN Abs [23]. Takeshita and colleagues have provided a possible explanation based on their demonstration that CEN C (but not CEN A or B), another target of the centromeric AC-3 pattern in HEp-2 cells, and Trim21 are co-expressed in salivary glands from SjS patients [24]. However, as anti-Trim21 detection has been included later in our study, analysis bias cannot be excluded. The Scl-70 antigen is a C-terminal breakdown product of Topo I that is processed during necrosis by cathepsin L and caspases in epithelial cells [25,26] and the autoantibody response is driven by cryptic epitopes probably in relation to severe micro-vasculopathy [27]. The presence of anti-Topo I Abs is associated with a specific pattern (AC-29) in HEp-2 cells and a correlation exists between anti-Topo I Ab levels and disease severity [28]. In our study, anti-Topo I Abs can be detected with near perfect performance ($K=0.94$) when considering the SSc immunodot and Luminex® based-assay within the SSc subgroup, which was not the case in the other subgroups ($0.65 < K < 0.73$). Even more striking, concordance between SSc immunodot (and Luminex® based-assay) with the AC-29 specific IIF pattern in HEp-2 cells was restricted to patients with SSc although Cohen's kappa coefficient remains moderate ($K=0.62$). One explanation comes from the fact that the pattern linked to the presence of anti-Topo I (AC-29) is quite heterogeneous and can present difficulties in recognition [13]. That can explain discrepancies between studies comparing identification assays and IIF patterns [29]. These data support the need to systematically reevaluate the IIF pattern when an anti-Topo I Ab is detected with a specific assay, and vice versa.

Combining two techniques (e.g., IIF and immunodot or IIF and the Luminex® based-assay) is recommended to improve the specificity for anti-Topo I Abs [30], and this was extended in our study to the set of antibodies detectable by the immunodot. This is of particular interest for anti-RNA Pol3 Abs detected in SSc that have the peculiarity to recognize a multi-molecular complex leading to important discrepancies when comparing specific assays (e.g., immunodot assays or line blot) [31]. Indeed, combining

an anti-RNA Pol3 positivity in SSc immunodot with the AC-4 pattern at high titer ($\geq 1/1280$) in HEp-2 cells allows an improvement in the Odds ratio from 2.3 (IC95: 0.9-5.9, $p=0.07$) to 10.2 (IC95: 1.3-78.0, $p=0.02$) when the SSc immunodot detection was coupled with IIF (IC95: 1.3-78, $p=0.02$).

Similarly, anti-fibrillarin Abs in SSc recognized a highly conserved protein that is a component of a multi antigenic complex including U3 ribonucleoprotein (U3-RNP, 34 kDa) present in the nucleoli [32] and first described as a clumpy nucleolar pattern in HEp-2 cells using sera from SSc patients [33]. Despite a clinical importance already documented for diagnostic evaluation and management [10,34], routine laboratory identification of this Ab remains limited due to technical limitations. Indeed, fibrillarin was particularly difficult to detect as initially reported when using an immunoprecipitation (IP) approach [35,36] and it was further established that performances of IP and SSc immunodot were similar in detecting anti-fibrillarin Abs [36]. In our study, association between SSc and anti-fibrillarin Ab detection was retrieved (OR=4.7, $p=0.04$) when SSc immunodot positivity was associated with a nucleolar pattern (AC-8-10) at high titer ($\geq 1/1280$) in HEp-2 cells.

For anti-Th/T0, anti-PM-Scl100, and PM-Scl75 the rarity of these Abs in our SSc cohort may explain that combining SSc immunodot with IIF detection improves the OR and PPV for these parameters but without reaching statistical significance for SSc association. Raising the question of the specificity of the anti-Ku and anti-U1 RNP Abs is difficult, or even utopian, because of the low number of positive samples in our cohort and the fact that these Abs are known to be associated with overlapping syndromes [37]. These overlap syndromes were included in the SSc or in the AD subgroups of our study, according to whether they reach or not the definition of SSc. However, these Abs seem to be rarely present in the Other disease group and are easy to correlate to their evocative HEp-2 cells pattern (AC-5 and AC-8-10).

5. Conclusion

In conclusion, at the time of diagnosis of SSc, it has been shown that the detection of associated autoantibodies provides an additional argument in very early SSc, disputed cases and borderlines forms to support the diagnosis. From this perspective, the

immunodot technique allowing the simultaneous detection of 11 scleroderma autoantibodies is therefore a technique of choice, widely adopted in laboratories. Unfortunately, this technique has drawbacks because the reading is subjective and can easily give false positives if not quantified as performed in our study. In addition, our work shows SSc immunodot presents good performance in detecting the most frequent SSc Abs (anti-CENP-A / B and Topo I) and has the advantage of detecting double positives anti-CEN A and CEN B (highly specific for SSc). Regarding minor SSc Abs (group 2), coupling IIF determination in HEp-2 cells with their detection on immunodot improves significantly result specificity for SSc, which was not the case when regarding the main SSc-associated clinical symptoms. Thus, coupling IIF determination in HEp-2 cells with the detection of 11 mutually exclusive and sometimes rare autoantibodies makes such association a strategy of choice to improve the rapidity and reliability of the results provided and therefore the usefulness of this test in clinical diagnosis of SSc.

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6. **Table 1:** Characteristics of patients with systemic sclerosis (SSc) included in the study. Abbreviations: CEN: centromere A and B; Topo I: topoisomerase I/Scl70; RNA Pol3: RNA Polymerase III; Fib: fibrillarin.

	All n=232	CEN A+B n=105	Topo I n=51	RNA Pol3 n=22	Fib n=18	Ku n=5	PM-Scl 75 n=8	PM-Scl 100 n=13	Th/T0 n=7	U1-RNP n=7	Trim21 n=27
Age (yrs) mean ± sd	50 ± 14.5	59.8 ± 13.4	56.6 ± 13.6	59.5 ±17.6	52.3 ± 16.6	63.5 ± 13.1	58.2 ± 14.0	54.9 ± 20.5	46.0 ± 10.7	46.8 ± 15.4	59.9 ± 14.8
Sex (F/M)	189/4 3	93/12	40/11	12/10	15/3	4/1	8/0	11/2	4/3	7/0	24/3
Limited cutaneous	75.3%	83.5%	58.3%	60%	66.7%	100%	75%	91.7%	66.7%	25%	84%
Diffuse cutaneous	18.3%	7.8%	37.5%	35%	33.3%	0	25%	8.3%	16.7%	75%	12%
Sine scleroderma	6.4%	7.8%	4.2%	5%	0	0	0	0	16.7%	0	4%
Rodnan's score (mean ± sd, (n))	7.6 ± 8.9 (87)	3.1 ± 4.3 (39)	11.1 ± 10.8 (22)	17.4 ± 10.4 (9)	5.2 ± 9.2 (7)	NA (1)	18 ± 11 (5)	2.7 ± 1.1 (5)	NA (2)	NA (1)	10 ± 4 (5)
Raynaud	93.2%	97.1%	95.8%	85%	94.4%	50%	75%	84.6%	83.3%	100%	92.3%
Telangiectasia	37.8%	49.5%	25.5%	20%	22.2%	0	71.4%	54.5%	16.7%	40%	29.7%
Interstitial lung disease	27%	9.7%	53.2%	30%	38.9%	50%	37.5%	50%	28.6%	60%	46.2%
Pulmonary arterial hypertension	7.3%	8.7%	4.2%	0	11.1%	0	12.5%	8.3%	0	0	23.1%
Pericarditis	7.2%	2%	11.1%	16.7%	25%	50%	12.5%	0	0	0	0
Muscular involvement	12.3%	8%	12.5%	30%	11.1%	0	37.5%	23.1%	28.6%	0	15.4%
Joint involvement	44.5%	45.5%	33.3%	50%	50%	75%	25%	23.1%	28.6%	66.7%	51.9%
Gastrointestin al involvement	53.6%	48.5%	60.4%	55%	55.6%	50%	50%	53.8%	42.9%	60%	69.2%
Scleroderma Renal crisis	2.8%	0	2.1%	10.5%	17.6%	0	0	0	0	20%	3.8%
Cancer	9.5%	8.8%	6.4%	15%	11.1%	0	0	8.3%	0	0	23.1%

7. Figures

Figure 1: Flow chart of the retrospective cohort study performed between July 2016 and July 2020 (n=1689). SSc immunodot positive samples coupled to indirect immunofluorescence (IIF) assay on HEp-2 cells (n=422) were considered and further analyzed when at least one parameter of the multi-antigenic SSc immunodot had an estimated arbitrary value ≥5 (D-Tek), when tested for the first time, and when clinical

information was available. Abbreviations: SSc: Systemic sclerosis; AD: autoimmune disease other than SSc; Other: non-autoimmune disease patients.

Figure 2: Multi-antigenic SSc immunodot results among patients with Systemic sclerosis (SSc; n=232), autoimmune disease (AD; n=91) other than SSc, and non-autoimmune disease (Other; n=48), which were tested positive for at least one parameter. **A:** Prevalence of the 11 associated SSc autoantibodies among the three disease subgroups at manufacturer's cut-off (≥ 5 arbitrary units [UA]). **B:** Quantitative analysis of SSc specific (Major Abs) anti-CEN A, anti-CEN B and anti-Topo I Abs. **C:** Quantitative analysis of the 8 remaining SSc immunodot parameters (Minor Abs). Intergroup comparison was performed using a Chi² 2x3 (A) or multiple Kruskal-Wallis nonparametric test with Dunn's adjustment (B, C) and *p* values are indicated when under 0.05. Abbreviations: CEN: centromere; Topo I: topoisomerase I/Scl70, RNA Pol3: RNA Polymerase III; Fib: fibrillar.

Figure 3: Anti-centromere (CEN) A, anti-CEN B, and anti-Topoisomerase (Topo) I immunodot performances. **A:** qualitative performance evaluation for anti-CEN A antibodies (Abs) tested with the Systemic sclerosis (SSc) immunodot as compared to the other specificities evaluated on the immunodot, AC-3 (centromere) and AC-29 (Topo I) pattern in HEp-2 cells, and multiplex immunofluorescence assay (Luminex®, Bio-Plex). **B:** qualitative performance evaluation for anti-CEN B Abs. **C:** qualitative performance evaluation for anti-Topo I Abs. The percentage of agreement to evaluate performance between assays (A-C) was calculated by determining the Kappa coefficient of Cohen (see material and methods for interpretation). **D:** Receiver operating characteristic (ROC) curve analysis of anti-CEN A Abs analyzed by SSc immunodot *versus* anti-CEN B Abs analyzed by Luminex® assay. **E:** ROC curve analysis of anti-CEN B Abs analyzed by SSc immunodot *versus* anti-CEN B Abs analyzed by Luminex® assay. **F:** ROC curve analysis of anti-Topo I Abs analyzed by SSc immunodot *versus* anti-Topo I Abs analyzed by Luminex® assay. Abbreviations: IIF: indirect immunofluorescence; AUC: area under the curve; AD: autoimmune disease other than SSc; Other: non-autoimmune disease patients.

Figure 4: Coupling indirect immunofluorescence (IIF) in HEp-2 cells with the SSc minor immunodot parameters anti-RNA Pol3, anti-U1 RNP, and anti-fibrillarin [Fib] antibody (Ab) improves immunodot performance for SSc. **A/B:** Characteristics of the restricted anti-nuclear Abs (anti-RNA Pol3 and anti-U1 RNP) on nuclear and nucleolar HEp-2 cell patterns (**A**) and the inverse of dilutions of the nuclear staining (**B**). **C/D:** Characteristics of the restricted anti-nucleolar Abs (anti-Fib) in nuclear and nucleolar HEp-2 cell patterns (**C**) and the inverse of dilutions of the nucleolar staining (**D**). Inverse dilutions are indicated from 1/160 to >1/5120. Abbreviations: Hom: homogeneous pattern (AC-1 according to the international consensus on antinuclear antibody pattern [ICAP]); CEN : centromere pattern (AC-3); Fine: fine speckled pattern (AC-4); Large: large speckled pattern (AC-5); Topo I: Topo I-like pattern (AC-29); Nuc : nucleolar pattern; AD : Autoimmune disease other than SSc; Other: non-autoimmune disease patients.

Figure 5: Coupling indirect immunofluorescence (IIF) in HEp-2 cells with the immunodot research for anti-Ku, anti-PM-Scl 75, anti-PM-Scl 100 and anti-Th/T0 antibodies (Abs) improves immunodot performance for autoimmune diseases (AD) at border with systemic sclerosis (SSc). **A/B:** Characteristics of the combined anti-nuclear and anti-nucleolar Abs tested on SSc immunodot as compared to nuclear and nucleolar HEp-2 cell patterns (**A**) and the inverse of dilutions of the nuclear staining (**B**). Inverse dilutions are indicated from 1/160 to >1/5120. Abbreviations: Hom: homogeneous pattern (AC-1); CEN: centromere pattern (AC-3); Fine: fine speckled pattern (AC-4); Large: large speckled pattern (AC-5); Topo I: Topo I-like pattern (AC-29); Nuc: nucleolar pattern; Fib: fibrillarin; Other: non-autoimmune disease patients.

Figure 6: Individual performance of the 11 antigenic targets tested on the SSc immunodot among patients with Systemic sclerosis (SSc), autoimmune disease (AD) other than SSc, and non-autoimmune disease (Other). **A/B:** Odds ratio (OR) and p-value for the occurrence of the 11 antigenic targets present on the multi-antigenic SSc immunodot when tested alone (**A**) or when coupled with indirect immunofluorescence (IIF) in HEp-2 cells (**B**). **C/D:** Impact of coupling multi-antigenic SSc immunodot with IIF in HEp-2 cells in terms of positive predictive value (PPV, **C**) or negative predictive value (NPV, **D**).

Supplemental Figure 1: Individual odds ratio (OR) of the 11 autoantibodies tested for three main SSc features; OR for Raynaud's phenomenon (A), interstitial lung disease (B), esophageal dysfunction or manifestations (C), figured in black when tested only with immunodot and in blue when coupled with indirect immunofluorescence (IIF) o HEp-2 cells concordant pattern.

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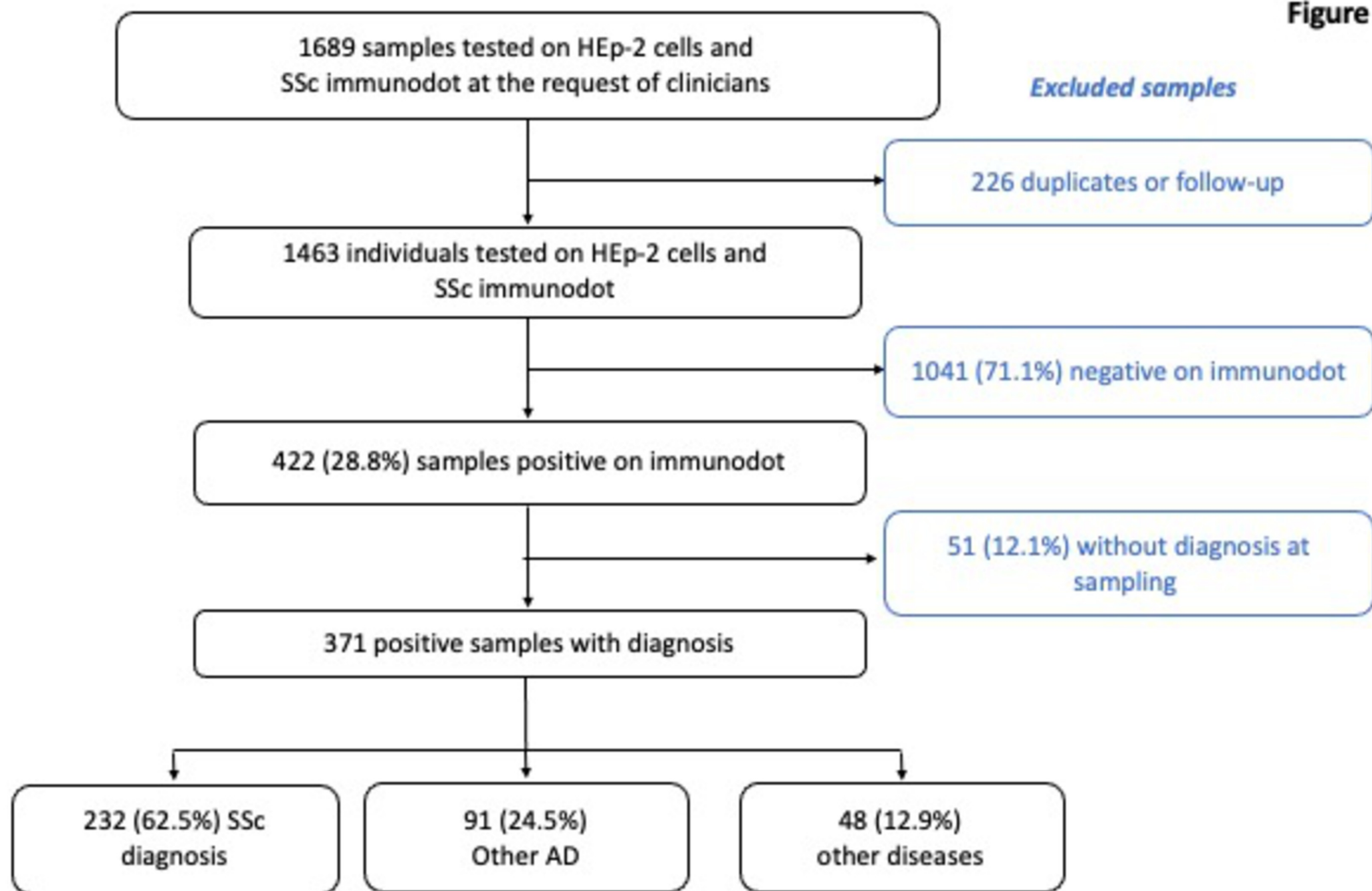
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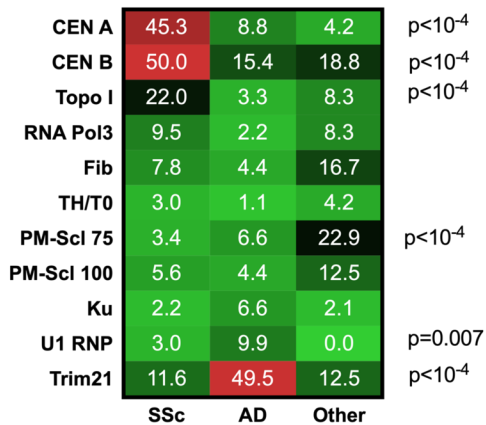
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Figure 1

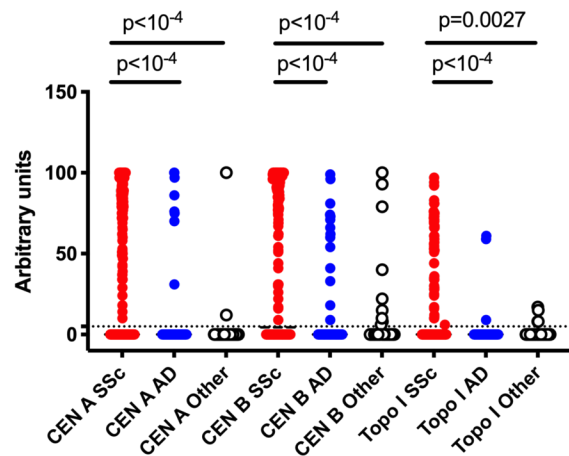


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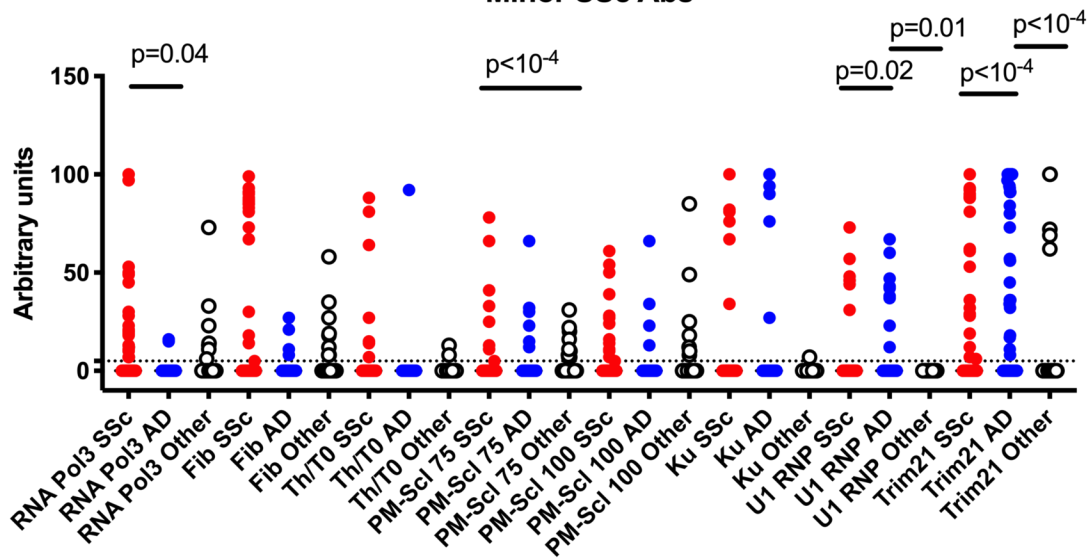
Prevalence (%)

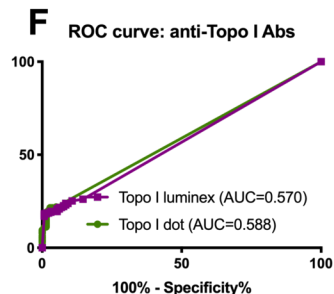
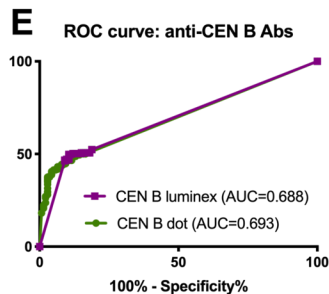
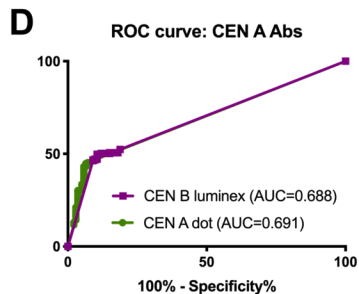
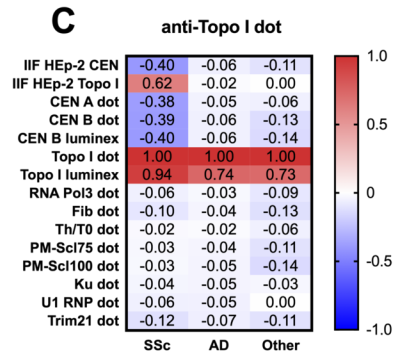
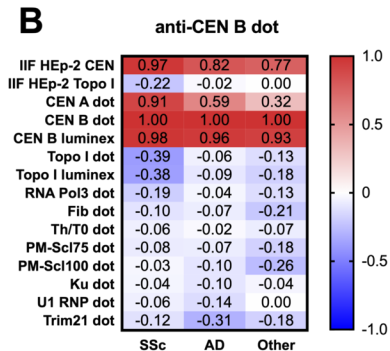
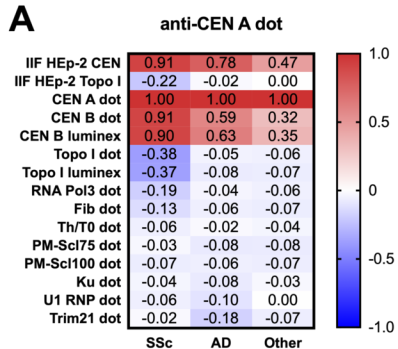
**B**

Major SSc Abs

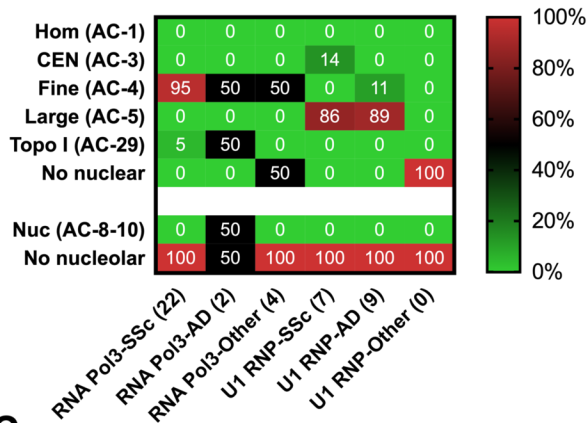
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Minor SSc Abs

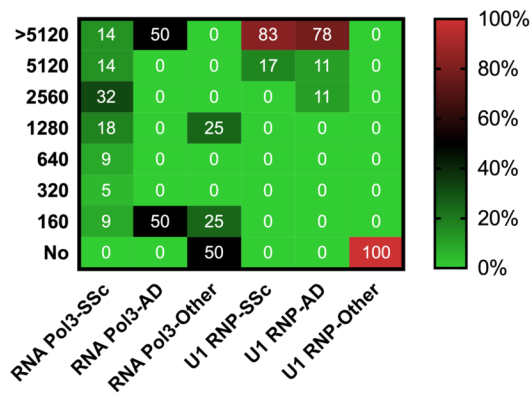




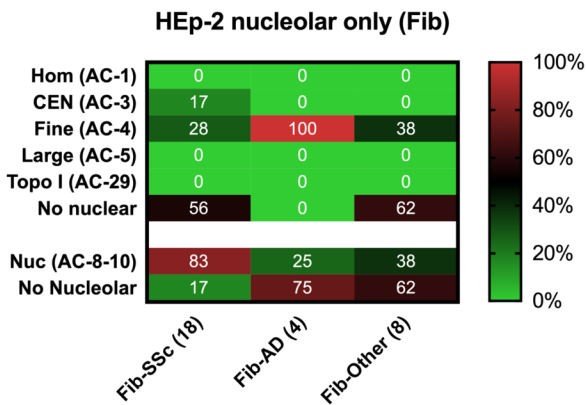
A Hep-2 nuclear only (RNA Pol3 & U1 RNP)



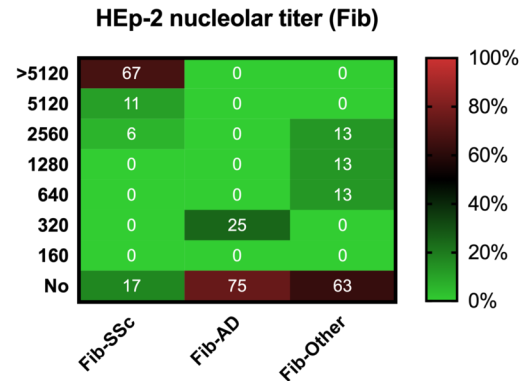
B Hep-2 nuclear titer (RNA Pol3 & U1 RNP)

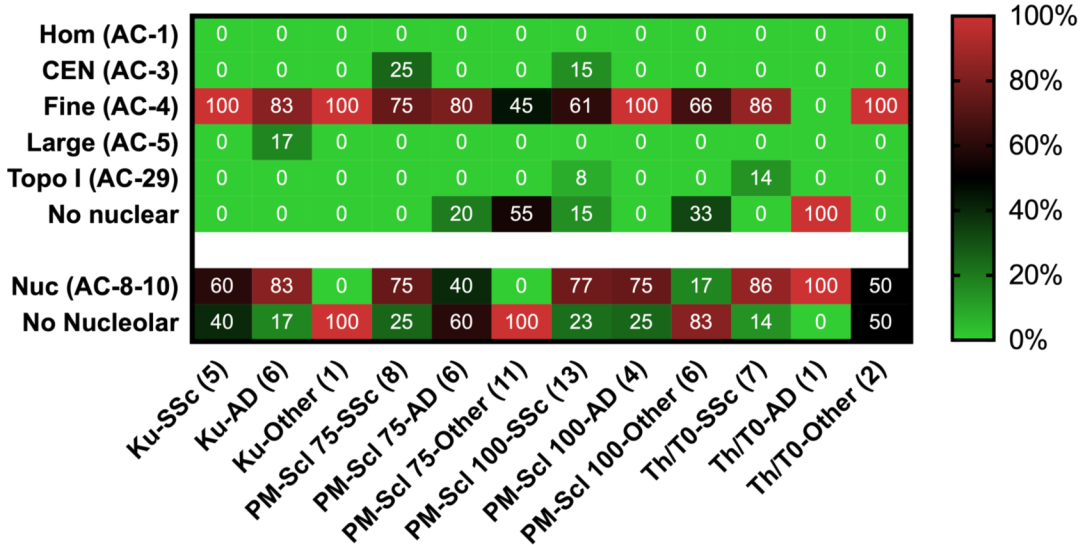


C Hep-2 nucleolar only (Fib)



D Hep-2 nucleolar titer (Fib)



A**HEp-2 nuclear & nucleolar pattern****B****HEp-2 nuclear & nucleolar titer**