

Longitudinal analysis of ANA in the Systemic Lupus International Collaborating Clinics (SLICC) inception cohort

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**Longitudinal Analysis of ANA in the Systemic Lupus
 International Collaborating Clinics (SLICC) Inception Cohort**

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3 **1 Longitudinal Analysis of ANA in the Systemic Lupus International Collaborating Clinics**
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6 **(SLICC) Inception Cohort**

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111 All authors were involved in the concept and design, data analysis and interpretation, and editing
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46
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50 51 159 **Data Sharing Statement**

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3 160 All data relevant to the study are included in the article or uploaded as supplementary
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5 161 information.

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10 163 **Patient and Public Involvement statement**

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14 166 plans of our research.
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ABSTRACT

Objectives: A perception derived from cross-sectional studies of small SLE cohorts is that there is a marked discrepancy between antinuclear antibody (ANA) assays, which impacts on clinician's approach to diagnosis and follow-up. We compared three ANA assays in a longitudinal analysis of a large international incident SLE cohort retested regularly and followed for five years.

Methods: Demographic, clinical, and serological data was from 805 SLE patients at enrolment, year 3 and 5. Two HEp-2 indirect immunofluorescence assays (IFA1, IFA2), an enzyme-linked immunosorbent assay (ELISA), and SLE-related autoantibodies were performed in one central laboratory. Frequencies of positivity, titres/units, and IFA patterns were compared using McNemar, Wilcoxon, and kappa statistics, respectively.

Results: At enrolment, ANA positivity ($\geq 1:80$) was 96.1% by IFA1 (median titre 1:1280 [IQR 1:640-1:5120]), 98.3% by IFA2 (1:2560 [IQR 1:640-1:5120]), and 96.6% by ELISA (176.3AU [IQR 106.4-203.5]). At least one ANA assay was positive for 99.6% of patients at enrolment. At year 5, ANA positivity by IFAs (IFA1 95.2%; IFA2 98.9%) remained high, while there was a decrease in ELISA positivity (91.3%, $p < 0.001$). Overall, there was $>91\%$ agreement in ANA positivity at all time points and $\geq 71\%$ agreement in IFA patterns between IFA1 and IFA2.

Conclusion: In recent-onset SLE, three ANA assays demonstrated commutability with a high proportion of positivity and titres/units. However, over five years follow-up, there was modest variation in ANA assay performance. In clinical situations where the SLE diagnosis is being considered, a negative test by either the ELISA or HEp-2 IFA may require reflex testing.

Keywords: Antinuclear antibodies, Systemic Lupus Erythematosus, longitudinal, performance, immunoassays, ELISA

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2
3 **191 INTRODUCTION**

4 192
5 193 Antinuclear antibody (ANA) testing has an integral approach to accurately diagnose and classify
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8 194 SLE (1). A systematic literature review and meta-regression of indirect immunofluorescence
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10 195 assays (IFA) reported high sensitivity (97.8%) for SLE diagnosis at a titer of $\geq 1:80$ (2). This
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12 196 presaged the decision to include a positive ANA at that titer on HEp-2 cell IFA “or an equivalent
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14 197 positive test on other diagnostic platforms” occurring at least once as an entry criterion for the
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16 198 2019 European League Against Rheumatism/American College of Rheumatology
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18 199 (EULAR/ACR) SLE Classification Criteria (3, 4).
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23 201 Previous longitudinal examinations of ANA and SLE-related autoantibodies suggest that a
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25 202 patient’s ANA status can change from positive to within the normal range and vice versa during
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27 203 the disease course (2, 5-16). However, these studies have typically been limited to small, single
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29 204 center cohorts with incomplete disease characterization, short follow-up, and/or using outdated
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31 205 assays with conflicting results. The factors influencing changes in ANA have also not been
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33 206 thoroughly studied. Taken together, this has left clinicians with uncertainty about the value and
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35 207 interpretation of ANA testing in making a diagnosis of, or classifying, SLE. In addition, the
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37 208 clinically actionable value of repeat ANA testing once a diagnosis of SLE is established requires
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39 209 clarification (17, 18).
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44 211 Much of the confusion and debate on the clinical utility of ANA testing in SLE is related to
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46 212 reported variations in HEp-2 IFA assay performance in cross-sectional cohorts (19-22), and
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48 213 some have questioned whether the ANA IFA should continue to be the “gold standard” screening
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50 214 test (23-25). For instance, in a cross-sectional study, Pisetsky et al. tested the same sera using
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3 215 different ANA assays (e.g., IFA, enzyme-linked immunosorbent assay [ELISA], and multiplex
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5 216 bead assay) (21) and reported that the frequency of an ANA test within normal reference range in
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7 217 SLE patients with disease duration ranging from 0.1 to 33.4 years varied from 4.9%–22.3%.
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10 218 Further, it has been proposed that the IFA could be replaced or complemented by newer
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12 219 generation solid phase multi-analyte immunoassays (SPMAI) such as ELISA and/or addressable
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14 220 laser bead immunoassays (ALBIA) (24-26). A recent systematic review and meta-regression
15
16 221 analysis of ANA testing in >13,000 SLE patients with disease duration ranging from 0–17 years
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18 222 reported that only ~2.5% of these patients had an IFA ANA <1:80 (2), although a higher
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20 223 prevalence of ANA within the normal reference range has been reported in other cohorts
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22 224 including the Systemic Lupus International Collaborating Clinics (SLICC) Inception Cohort
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24 225 (6.2% were <1:160 at inception) (27).
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31 227 The primary goal of this study was to gain a more thorough understanding of ANA detection and
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33 228 its clinical value by comparing the performance of three currently available ANA assays in a
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35 229 longitudinal analysis (at least 5 years) of a large multinational SLE inception cohort.
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40 231 **METHODS**

41 42 232 *Study Population*

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44 233 Between 1999 and 2011, SLICC (<https://sliccgroup.org>) (28) enrolled 1827 patients fulfilling the
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46 234 1997 Updated ACR SLE Classification Criteria for definite SLE (29) within 15 months of
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48 235 diagnosis from 31 medical centres in 11 countries. Sera, clinical and demographic data were
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50 236 collected at enrolment and annually thereafter. Of the 1827 patients, 1432 (78.4%) were followed
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53 237 for ≥4 years; of these 1432 patients, we included the 805 patients who provided an enrolment
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3 238 and two additional serum samples within five years of enrolment, with the third sample being ≥ 4
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5 239 years after enrolment. The study was approved by the Institutional Review Board at each
6
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15 243 *ANA and Autoantibody Testing*

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17 244 Aliquots of sera were obtained from the 805 patients in the SLICC Inception Cohort at three time
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19 245 points: 1) enrolment (sample #1), 2) two to four years after enrolment (sample #2), and 3) four to
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21 246 10 years after enrolment (sample #3). Hereafter, samples #1 – 3 are referred to as enrolment,
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23 247 year 3, and year 5, respectively. Samples were stored at -80°C until required for immunoassays
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25 248 and analyzed centrally at MitogenDx Laboratory (Calgary, Canada). Three US Food and Drug
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27 249 Administration (FDA)-approved and Conformitè Européenne (CE) marked ANA tests were
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29 250 used, including two HEp-2 IFA, IFA1 (Bio-Rad Laboratories, Hercules, USA) and IFA2
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31 251 (NovaLite, Werfen, San Diego, USA), and an ELISA (Werfen, San Diego, USA). In accordance
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33 252 with the manufacturers' directions, a positive test was defined as a titer of $\geq 1:80$ for IFA1 and
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35 253 IFA2 (titre $< 1:80$ is considered normal range) and ≥ 20 absorbance units (AU) for ELISA. IFA1,
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37 254 IFA2, and ELISA were tested on the full patient cohort ($n=805$) sera from all three time points.
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39 255 IFA results (titres and patterns) were initially read by an automated digital IFA microscope and
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41 256 then checked manually by a technologist with 30 years of experience. ANA IFA patterns were
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43 257 classified according to the new International Consensus on ANA Patterns recommendations
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45 258 (<http://www.anapatterns.org/index.php>) (30). Quality control was performed by repeating all
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47 259 ANA results that were within the normal range and a random selection of ANA-positive samples
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260 to ensure inter-test reliability. SLE-related autoantibodies (**Supplemental Table 1**) were also
261 performed on each patient at enrolment, year 3 and 5.

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263 *Clinically Defined Samples*

264 Demographic and clinical data (**Supplemental Table 2**) at enrolment included age, sex, disease
265 duration, race/ethnicity, nephritis (fulfilling the ACR criterion for renal disease or based on a
266 renal biopsy), ACR Classification Criteria, Systemic Lupus Erythematosus Disease Activity
267 Index – 2000 (SLEDAI-2K), SLICC/ACR Damage Index (SDI), and medication use (current and
268 ever use of glucocorticoids, antimalarials, and immunosuppressives, including biologics). We
269 also collected longitudinal data on nephritis, SLEDAI-2K, SDI, and medications.

270

271 *Statistical analysis*

272 Demographic, clinical, and serological characteristics were described using summary statistics.
273 Changes over time in demographic and clinical features were described using differences in
274 means or proportions, with 95% confidence intervals (CI). As our analysis used a subgroup of
275 the larger SLICC cohort based on sera availability, we compared the enrolment characteristics of
276 the 805 patients included in this study with the 627 patients who were followed for ≥ 4 years but
277 were not included as three serial serum samples were unavailable. We also compared the
278 characteristics of the 781 patients providing the third serum sample 4-7 years after enrolment
279 with the 24 patients providing the third serum sample 8-10 years after enrolment.

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281 We assessed the frequency of ANA positivity and titre at each time point. Using the paired
282 McNemar's test, we calculated changes in ANA positivity between enrolment and year 5 for

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3 283 each test and the inter-test agreement in ANA positivity between tests at each time point. A
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5 284 histogram with a curve of best fit line was used to plot the changes in distribution of titres and
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8 285 units over time were compared using the Wilcoxon signed rank test for paired data. We
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10 286 examined the frequency of each ANA pattern and how many patients retained their HEp-2 IFA
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12 287 pattern over the three serial samples. ANA patterns were further categorized into three groups: 1)
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14 288 isolated nuclear (AC 1-14, 29), 2) isolated cytoplasmic and/or mitotic (CMP, AC 15-28), and 3)
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16 289 mixed nuclear and CMP patterns. Agreement between IFA1 and IFA2 ANA titres and patterns
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19 290 was assessed using the weighted and unweighted kappa (κ) statistic, respectively. Established
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21 291 SLE-related autoantibody profiles of patients with an ANA result within the normal range on
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23 292 IFA1, IFA2, or ELISA alone, on two of three assays, and on all three assays at enrolment and
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25 293 year 5 were examined to understand which autoantibodies were not being captured by the ANA
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28 294 screening assays. Statistical analysis was performed using Stata 15.1 (StataCorp, College Station,
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31 295 TX, USA).

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35 297 **RESULTS**36
37 298 *Study Population*

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40 299 Eight hundred and five SLE patients were included. The mean time from disease diagnosis to
41
42 300 enrolment was 0.58 years (standard deviation [SD] 0.49); the mean time between the enrolment
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44 301 and the year 3 sample was 2.8 years (SD 0.8) and between the enrolment and the year 5 sample
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46 302 was 5.0 years (SD 1.1). Patients had a mean age at diagnosis of 35.2 years (SD 13.6), 88.7%
47
48 303 (714/805) were female and 47.7% (384/805) were of race/ethnicity other than White (**Table 1**).
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50 304 From enrolment to year 5, the prevalence of lupus nephritis increased by 7.7% [95%CI: 5.7%,
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52 305 9.7%], mean SLEDAI-2K decreased by 2.3 [95%CI: 1.9, 2.7], and mean SDI increased by 0.52
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3 306 [95%CI: 0.43, 0.62]. There were significantly fewer patients on glucocorticoids (69.6% vs
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5 307 56.8%, difference -12.8% [95%CI: -16.5%, -9.1%]) and more patients on antimalarials (70.1%
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7 308 vs 79.4%, difference 9.3% [95%CI: 5.9%, 12.7%]) or immunosuppressants (41.0% vs 50.8%,
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9 309 difference 9.8% [95%CI: 6.1%, 13.5%]). The frequency of most SLE-related autoantibodies
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11 310 decreased at year 5.
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17 312 The enrolment characteristics of the 805 patients included in our study were similar to the 627
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19 313 patients who provided ≥ 4 years of data but did not have three available serial serum samples
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21 314 (**Supplemental Table 3**). However, there was a higher proportion of Asian (18.8% (95%CI:
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23 315 15.3, 22.2) and lower proportion of Hispanic participants (-20.6% (95%CI: -24.5, -16.8) in the
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25 316 study cohort compared to the cohort not providing serial samples. The enrolment characteristics
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27 317 of the 781 patients whose year 5 sample was collected between years 4 and 7 were similar to the
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29 318 24 patients whose year 5 sample was collected between years 8 and 10 (**Supplemental Table 4**).
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34 320 *ANA Positivity and Agreement Among Different Assays Over Time*

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36 321 At enrolment, the frequency of ANA positivity by IFA1, IFA2, and ELISA was high (96.1%
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38 322 [95%CI: 94.6-97.3%], 98.3% [95%CI: 97.1-99.0%], and 96.6% [95%CI: 95.2-97.7]),
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40 323 respectively (**Figure 1**) and 99.6% (802/805) of patients had ≥ 1 positive ANA of $\geq 1:80$. An
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42 324 additional five (0.6% incremental effect), three (0.5%), and two patients (0.4%) at enrolment,
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44 325 year 3, and year 5 visits, respectively, would be ANA positive on the ELISA, but within the
45
46 326 normal range for both IFA1 and IFA2. There was no significant change in ANA positivity at
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48 327 enrolment compared to year 5 for IFA1 or IFA2. However, ANA positivity by ELISA decreased
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50 328 significantly from enrolment to year 5 (difference -5.3% (95%CI: -7.4, -3.3), $p < 0.001$) such that
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3 329 91.3% (735/805) of patients were positive by year 5. Notably, 1.2% (10/805) of subjects were
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5 330 within the normal range at all three time points by ELISA compared to 0.9% (7/805) by IFA1
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7 331 and 0.1% (1/805) by IFA2. At all time points, no patients were classified as being within the
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9 332 normal range if all three of the assays were considered.

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14 334 Overall, the inter-test agreement for positivity between any pair of assays was >91% (**Table 2**).
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16 335 In cases where there was disagreement between IFA1 and IFA2, there was significant asymmetry
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18 336 (McNemar's test) such that most disagreements were due to more patients with an ANA by IFA1
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20 337 within the normal range and a positive ANA by IFA2 (-IFA1/+IFA2) rather than a positive ANA
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22 338 by IFA1 and an ANA within the normal range by IFA2 (+IFA1/-IFA2) for all three time points
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24 339 (**Supplemental Table 5**). Regarding the disagreements between IFA1 and ELISA, there was no
25
26 340 significant asymmetry until year 5 when there were more cases of disagreement due to +IFA1/-
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28 341 ELISA compared to -IFA1/+ELISA. For disagreements between IFA2 and ELISA, there was
29
30 342 significant asymmetry across all time points with more cases of +IFA2/-ELISA than -
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32 343 IFA2/+ELISA.

34 344 35 36 37 38 39 345 *ANA Titres/Units Among Different Assays Over Time*

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41 346 At enrolment, the median ANA titre/unit for IFA1, IFA2, and ELISA were 1:1280 (interquartile
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43 347 range (IQR) 1:640-1:5120), 1:2560 (IQR 1:640-1:5120), and 176.3 AU (IQR 106.4 AU-203.5
44
45 348 AU), respectively (**Figure 2**). The distribution of ANA titres was skewed to the left for all assays
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47 349 at enrolment (higher proportion of patients with very high ANA titres). Only a small proportion
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49 350 had ANA titres of 1:80 to 1:160 at enrolment (IFA1 10.4% [84/805] and IFA2 8.1% [65/805]).
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51 351 The median titres/units at year 5 were significantly lower compared to enrolment for IFA1
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352 (1:640 (IQR 1:320-1:2560), paired Wilcoxon signed rank $p < 0.0001$, a change in one dilution
353 step) and ELISA (157.3 CU (IQR 66.14 CU- 200.65 CU), $p < 0.0001$). There was good
354 agreement between IFA1 and IFA2 titres at enrolment, 84.9% (95%CI: 82.2-87.3) agreement,
355 $k = 0.49$ (95%CI: 0.45-0.53); at year 3, 81.1% (95%CI: 78.2-83.7%) agreement, $k = 0.39$ (95%CI:
356 0.35-0.43%); and at year 5, 82.0% (95%CI: 79.1-84.6%) agreement, $k = 0.41$ (95%CI: 0.37-
357 0.45%).

359 *ANA Patterns Among Different Assays Over Time*

360 The most common ANA IFA pattern was an isolated nuclear staining pattern for IFA1 (62.1%-
361 68.7%) and IFA2 (59.3%-62.1%) at all visits (**Table 3**). The top three individual IFA patterns for
362 both IFA1 and IFA2 were AC-1 (homogeneous), AC-4 (nuclear fine speckled), and AC-5
363 (nuclear large speckled) (**Supplemental Figure 1**). There was fair-to-moderate agreement
364 between IFA1 and IFA2 ANA IFA staining patterns at enrolment, (74.0% [95%CI 70.7-77.0]
365 agreement, $\kappa = 0.46$ [95%CI 0.39-0.53]), year 3, (71.4% [95%CI 68.0-74.6], $\kappa = 0.39$ [95%CI
366 0.33-0.46]), and year 5, (71.0% [95%CI 67.7-74.2], $\kappa = 0.39$ [95%CI 0.33-0.46]).

368 *ANA Patients Within the Normal Range and Seroconversion*

369 At enrolment and year 5, 8 and 20 patients were within normal range by IFA1 & ELISA, 3 and 4
370 patients by ELISA & IFA2, and 8 and 6 patients by IFA1 and IFA2 (**Table 4**). When examining
371 the autoantibody profiles of patients whose ANA were within normal range at enrolment or year
372 5, depending on the assay 38.7%-53.8% had no detectable SLE-related autoantibodies. Anti-
373 Ro52/TRIM21 and anti-SSA/Ro60, the former not detectable by HEp-2 IFA and the latter does
374 not have a clearly established IFA pattern, were the most frequent autoantibodies detected when

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3 375 the ANA test was within normal range. Seroconversion from ANA positive to normal range (titre
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5 376 <1:80) from enrolment to year 5 was observed in 4.8% (39/805) of patients using IFA1, 1.1%
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7 377 (9/805) using IF2, and 8.7% (70/805) using ELISA. The median titre of ANA at enrolment prior
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9 378 to seroconversion was low (IFA1 1:160 [IQR 1:80-1:640]), IFA2 1:320 [IQR 1:160-1:2560], and
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11 379 ELISA 61.5 CU [IQR 20-158]). Among those who were originally anti-dsDNA positive at
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13 380 enrolment (n=273), the frequency of ANA positivity was high at enrolment irrespective of the
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15 381 ANA assay (99.3-100.0%). At year 5, frequency of ANA positivity for these same patients,
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17 382 irrespective of their anti-dsDNA status at year 5, declined slightly using for the IFAs (IFA -2.2%,
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19 383 IFA2 -1.1%) and -4.8% for the ELISA (data not shown).
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25 26 385 **DISCUSSION**

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28 386 To our knowledge, this is the largest longitudinal, multinational study (805 patients and 2415
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30 387 serum samples) that compared the performance of different ANA assays in a well-characterized
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32 388 inception cohort of SLE patients. Our study was designed to overcome the limitations of prior
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34 389 reports that studied smaller cohorts and were historical and/or cross-sectional in nature. These
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36 390 data are timely given ANA test positivity is an entry criterion for the 2019 EULAR/ACR
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38 391 classification criteria for SLE (31, 32). We found that, regardless of the assay, almost all patients
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40 392 with recent onset SLE (802/805) had a positive ANA at enrolment on ≥ 1 assay, all were ANA-
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42 393 positive on ≥ 1 assay at least once across the five years, and the mean ANA titres/values were
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44 394 high. However, over the five years, some variation between ANA assay performance was
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46 395 detected, including a statistically significant decrease in ELISA ANA positivity and reduction in
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48 396 titres for IFA1 and ELISA.
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3 398 It has been suggested that the variation in performance between different ANA assays may be
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5 399 related to differences in laboratory techniques, equipment, inter-observer consistency and
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7 400 reagents (25, 33). However, in our study, all ANAs were performed and interpreted at one
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9 401 central laboratory by a highly experienced (30 years of experience) technician. Even after
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11 402 controlling for the impact of inter-laboratory and inter-observer variation, we still identified
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13 403 some significant inter-assay disagreement. Disagreement between ELISA and IFA is likely
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15 404 primarily due to factors intrinsic to the test platforms themselves. Unlike the IFA, the ELISA
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17 405 contains extracts of cell homogenates augmented by purified proteins derived from native and/or
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19 406 synthetic, recombinant sources (34). The composition of the different ELISA ANA preparations
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21 407 is diverse and dependent on the manufacturer as to which key target autoantigen(s) associated
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23 408 with autoimmune diseases are included and at what concentrations (34). ELISAs may also have
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25 409 decreased detection of ANA because of poor autoantibody binding, as some antigens may also
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27 410 bind to other targets in the same mixture, resulting in a masking effect. Furthermore, many
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29 411 autoantibody targets are components of macromolecular complexes where key epitopes may be
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31 412 hidden or masked (34). A thorough study of the affinity and avidity of the various autoantibodies
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33 413 would add useful understanding to the use of ANA ELISAs.
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415 Prior studies of more established SLE patients reported that as high as 30% have an ANA below
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43 416 the positive threshold (35). Over time, we observed a decrease in ANA positivity with ELISA, a
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45 417 decrease in ANA titres/values with IFA1 and ELISA, and decreased detection of specific
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47 418 autoantibodies. We postulate that factors such as disease activity and medication exposure
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49 419 influence ANA (36-39). However, the extent to which therapeutic interventions can alter ANA
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51 420 production, especially by long-lived plasma cells, remains to be proven, and the expression of
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3 421 other autoantibodies can occur following diagnosis, attributed to epitope spreading continuing
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5 422 despite therapy(39).
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10 424 Our study addresses important questions raised about the ANA in the 2019 EULAR/ACR SLE
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12 425 classification criteria (3, 4, 40), which require an “ever positive” ANA of $\geq 1:80$ by HEp-2 IFA
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14 426 or an equivalent test on another platform as an entry criterion for classification. For example, it is
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16 427 important to note that all subjects had at least one positive ANA at the 1:80 threshold over the
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18 428 five years of follow-up. The new criteria also state that a solid phase assay of at least equivalent
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20 429 performance can be used in place of the HEp-2 IFA, although a precise definition of ‘equivalent
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22 430 performance’ was not specified. Our results show that although some inter-assay disagreement
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24 431 exists between these three assays, >91% of recent-onset SLE patients will have a positive ANA
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26 432 using either HEp-2 IFA or ELISA, although titres decreased by year 5 for IFA1 and the ELISA.
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28 433 As expected from previous reports (20, 41), ELISA had the highest proportion of SLE patients
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30 434 with an ANA within the normal $<1:80$ reference range, and therefore, the ELISA used as a
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32 435 screening test may benefit from judicious reflex testing to the HEp-2 IFA. In turn, since the HEp-
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34 436 2 IFA can be negative when the ELISA is positive, the reciprocal reflex approach could be
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36 437 considered.
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44 439 Importantly, consistent with other studies and emerging recommendations on ANA testing (20,
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46 440 41), we demonstrated that a combination of two different ANA assays reduced the proportion of
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48 441 SLE patients with ANAs in the normal range; particularly when IFA2 was combined with
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50 442 ELISA. A combination of all three assays resulted in no patients who had an ANA within normal
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52 443 range at enrollment and two subsequent follow-up visits. This helps shed light on the question of
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3 444 the value of ANA testing to follow the clinical course of SLE, but more detailed follow-up
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5 445 studies evaluating disease activity and flares at follow-up visits in the context of ANA testing are
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7 446 still required. Health care providers should be aware of the technical issues for ANA assays used
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10 447 in their jurisdictions and recognize that different ANA assays or simply following
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12 448 manufacturer's recommended reference ranges might not be optimal in applying ANA testing
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14 449 results (42, 43). Additional longitudinal studies comparing other ELISAs and SPMAI such as
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16 450 other multiplex bead immunoassays and emerging ANA technologies are needed.
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21 452 Our study has some important strengths. To our knowledge, this is the largest review of ANA
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23 453 status in SLE patients with data collected longitudinally and in a protocolized fashion over a
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25 454 mean follow-up of five years. All ANA testing was conducted in an accredited central laboratory
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27 455 with stringent quality control. However, we acknowledge some important limitations. First, there
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29 456 may be a potential selection bias for SLE patients who are ANA positive to be enrolled into the
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31 457 SLICC cohort compared to patients in conventional clinical care. Second, as enrolment could
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33 458 occur up to 15 months after diagnosis (although mean disease duration at enrolment was 0.58
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35 459 years), most patients had already been exposed to ≥ 1 immunomodulatory medication by
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37 460 enrolment, which could potentially influence the ANA result. Third, although we showed that
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39 461 demographic and clinical characteristics of the cohort subset with three available serum samples
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41 462 were largely similar to the remainder of the cohort, our sample included a larger proportion of
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43 463 Asian and fewer Hispanic participants. While our sample was racially and geographically
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45 464 diverse, it is not known if our findings are generalizable to other SLE cohorts. Fourth, the
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47 465 duration of follow-up, although relatively long at five years, does not capture potential
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49 466 seroconversions or measure assay performance later in the disease. Last, there are >10 different
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3 467 ANA immunoassays in use world-wide and our study utilized three. Regrettably, some
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5 468 manufacturers declined to participate in this study. Hence, generalization to all ANA assays is
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8 469 not possible (42, 44).
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12 471 In conclusion, we demonstrated that early in their disease course almost all adult SLE patients
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14 472 had highly positive ANAs. However, as the disease progressed, we observed increased frequency
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16 473 of ANA within the normal range and decreased ANA titres/values by some assays likely related
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18 474 to differences in assay performance, medication exposure, decreased autoantibody responses
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20 475 over time, and lower disease activity. Combining ANA assays resulted in fewer patients that
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22 476 tested within normal range and no patients who tested within the normal range over the five
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24 477 years with all three assays. A clinical implication of this study is that for patients who have a
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26 478 moderate-to-high suspicion of SLE, especially those early in the disease course but without an
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28 479 established diagnosis, screening on both ELISA and HEp-2 IFA is warranted if one or the other
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30 480 provides results in the normal range. And given the rather modest changes in ANA frequency
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32 481 (and/or titers) observed in this longitudinal study of 5 years follow up, it is difficult to perceive
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34 482 of actionable clinical value of ANA IFA or screening ELISA test results over this time period
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36 483 once the diagnosis of SLE has been established. Since there are differences in the performance
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38 484 characteristics of individual ANA assays, clinicians need to be aware of the performance
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40 485 characteristics of the ANA test that their laboratories use. Future studies testing the comparative
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42 486 performance of other ANA immunoassays over time in large populations will help inform
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44 487 approaches to an earlier and more accurate diagnosis and classification of SLE.
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3 488 **Key Messages:**

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5 490 What is already known about this subject?

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8 491 • Cross-sectional data of small cohorts suggest significant variation in the performance of
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10 492 antinuclear antibody (ANA) assays from different manufacturers leaving clinicians
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12 493 uncertain about the use or value of ANA testing in making a diagnosis.

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17 495 What does this study add?

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19 496 • In a longitudinal analysis of well-characterized patients with incident systemic lupus
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21 erythematosus (SLE), almost all SLE patients early in disease had highly positive ANAs
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23 and no patients who tested within the normal range over 5 years of follow up with all
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27 three assays.
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31 500 • As the disease evolved over 5 years of follow-up, there was decreased frequency of
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34 501 positive ANAs (above the normal range) and decreased ANA titres by some assays.
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40 503 How might this impact on clinical practice or future developments?

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42 504 • In a patient without an established diagnosis of SLE and in whom the clinical suspicion
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44 505 for SLE is moderate to high, both IFA and ELISA should be performed if one or the other
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46 506 provides results in the normal range.
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508 TABLES

509 Table 1. Patient characteristics at enrolment and year 5 (n=805)

Characteristic	Enrolment	Year 5	Difference ¹ (95% CI)
Demographic and Clinical			
Mean age at dx, yrs (SD)	35.2 (13.6)		
Female, %	88.7		
Mean disease duration, yrs (SD)	0.58 (0.49)		
Mean number of ACR Criteria without ANA (SD)	3.9 (1.0)		
Ethnicity, %			
Asian	24.3		
African	13.5		
White	52.3		
Hispanic	6.3		
Other ethnicities ²	3.5		
Nephritis ³	28.9	36.6	7.7 (5.7, 9.7)
Mean total SLEDAI-2K (SD) ⁴	5.4 (5.3)	3 (3.5)	-2.3 (-2.7, -1.9)
Mean total SDI (SD) ⁵	0.34 (0.74)	0.86 (1.25)	0.52 (0.43, 0.62)
Medications			
Current, %			
Glucocorticoids	69.6	56.8	-12.8 (-16.5, -9.1)
Antimalarials	70.1	79.4	9.3 (5.9, 12.7)
Immunosuppressants	41.0	50.8	9.8 (6.1, 13.5)
Ever, %			
Glucocorticoids	81.5	87.3	5.8 (4.1, 7.6)
Antimalarials	76.6	91.1	14.4 (11.9, 17)
Immunosuppressants	43.9	66.3	22.5 (19.5, 25.5)
Autoantibodies, %			
dsDNA ⁶	34.2	29.1	-5.1 (-8.7, -1.6)
Ribosomal P	24.3	20	-4.3 (-7.8, -0.9)
Ro52/TRIM21	37.5	37.4	-0.1 (-3.4, 3.2)
SSA/Ro60	42.5	42	-0.5 (-3.7, 2.7)
SSB/La	20.7	16.3	-4.5 (-7.5, -1.5)
Sm	22.7	14.7	-8.1 (-11.1, -5.0)
U1RNP	28.2	23	-5.2 (-8.5, -2.0)
Histones	31.3	22.7	-8.6 (-12.1, -5.0)
Cardiolipin IgG/IgM ⁷	20.5	16.4	-4.1 (-7.7, -0.6)
β2GP1 IgG/IgM ⁷	19.8	12.9	-6.9 (-9.8, -4)
Lupus anticoagulant ⁸	20.6	16.7	-3.9 (-9.8, 2)
Abbreviations: ACR, American College of Rheumatology; ANA, anti-nuclear antibodies; β2GP1, β2-glycoprotein-1; CI, confidence interval; dx, diagnosis; dsDNA, double-stranded DNA; IgG/M, immunoglobulin G/immunoglobulin M; RNP, ribonucleoprotein; SD, standard deviation; SLEDAI-2K, systemic lupus erythematosus disease activity index-2000; SDI, SLICC Damage index; Sm, Smith; TRIM21, Tripartite Motif Protein (TRIM) 21; yrs, years.			
1. Difference between enrolment and year 5 visit;			
2. Other ethnicities include: Native North American, Native Hawaiian or other Pacific Islanders			
3. Nephritis defined as fulfilling the ACR criterion for renal disease or if a renal biopsy was performed prior to cohort entry			
4. Complete data available for n=793 patients			
5. Complete data available for n= 380 as the disease needs to be present for at least 6 months before the SDI can be calculated.			
6. Complete data available for n=798 patients			
7. Complete data available for n= 800			
8. Complete data available for n=282			

510 **Table 2. ANA inter-test percentage agreement among IFA1 (n=805), IFA2 (n=805), and**
 511 **ELISA (n=805)**
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	Enrolment (%)		Year 3 (%)		Year 5 (%)	
	IFA1	IFA2	IFA1	IFA2	IFA1	IFA2
IFA2	96.4% (94.9 -97.6)		95.2% (93.4-96.5)		95.5% (93.9-96.8)	
ELISA	94.8% (93.0-96.2)	95.7% (94.0-97.0)	91.2% (89.0-93.0)	92.5% (90.5-94.3)	91.4% (89.3-93.3)	91.2% (89.0-93.0)

Abbreviations: ANA, anti-nuclear antibodies; ELISA, enzyme-linked immunosorbent assay; IFA; indirect immunofluorescence assay.

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515 **Table 3. ANA patterns over time with indirect immunofluorescence assay (IFA) 1 (n=805)**
 516 **and IFA2 (n=805)**
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Pattern	Enrolment n (%)	Year 3 n (%)	Year 5 n (%)	Same ANA Pattern Over 5 years n (%)
IFA 1 Patterns				
Nuclear	481 (62.1)	519 (68.1)	526 (68.7)	305 (37.9)
Cytoplasmic +/- Mitotic	17 (2.2)	18 (2.4)	21 (2.7)	1 (0.1)
Mixed	276 (35.7)	225 (29.5)	219 (28.6)	81 (10.1)
IFA2 Patterns				
Nuclear	491 (62.1)	477 (60.4)	472 (59.3)	273 (33.9)
Cytoplasmic +/- Mitotic	9 (1.1)	6 (0.8)	4 (0.5)	0 (0.0)
Mixed	291 (36.8)	308 (38.8)	320 (40.2)	114 (14.2)
IFA1 and 2 agreement (k)				
Agreement (95%CI)	74.0 (70.7- 77.0)*	71.4 (68.0- 74.6)*	71.0 (67.7- 74.2)*	
Kappa (95%CI)	0.46 (0.39- 0.53)	0.39 (0.33- 0.46)	0.39 (0.33- 0.46)	
Abbreviations: ANA, anti-nuclear antibodies; IFA; indirect immunofluorescence assay. *p<0.0001 using unweighted kappa (k) statistics.				

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519 **Table 4. Autoantibodies detected in patients with an ANA that was within the normal range on IFA1, IFA2, ELISA, either**
 520 **alone, on two or all three assay at enrolment and year 5***
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% Autoantibodies	ELISA		IFA1		IFA2		IFA1&ELISA		ELISA and IFA2		IFA1&IFA2		All three assays	
	Enrolment (N=27)	Year 5 (N=70)	Enrolment (n=31)	Year 5 (n=39)	Enrolment (N=14)	Year 5 (N=9)	Enrolment (N=8)	Year 5 (N=20)	Enrolment (N=3)	Year 5 (N=4)	Enrolment (N=8)	Year 5 (N=6)	Enrolment (N=3)	Year 5 (N=3)
None detected	44.4	45.7	38.7	53.8	42.9	44.4	62.5	65.0	66.7	50.0	50.0	50.0	66.7	66.7
dsDNA ¹	7.7	5.7	6.7	5.1	0.0	11.1	0.0	0.0	0.0	0.0	0.0	16.7	0.0	0.0
Ribosomal P	3.7	11.4	6.5	10.3	7.1	11.1	0.0	10.0	0.0	25.0	0.0	16.7	0.0	33.3
Ro52/TRIM21	11.1	21.4	22.6	20.5	21.4	11.1	0.0	20.0	0.0	25.0	0.0	0.0	0.0	0.0
SSA/Ro60	7.4	12.9	25.8	10.3	21.4	11.1	0.0	5.0	0.0	0.0	12.5	0.0	0.0	0.0
SSB/La	7.4	7.1	0.0	5.1	0.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
Sm	3.7	4.3	6.5	2.6	0.0	11.1	0.0	0.0	0.0	25.0	0.0	0.0	0.0	0.0
U1RNP	3.7	7.1	0.0	5.1	0.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0
Histones	0.0	10.0	0.0	2.6	7.1	11.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Patients who were within the normal range for ANA at enrolment are not necessarily the same patients at year 5 and vice versa.
 Abbreviations: ANA, anti-nuclear antibodies; β 2GP1, β 2-glycoprotein-1; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay, IFA; indirect immunofluorescence assay; IgG/M, immunoglobulin G/immunoglobulin M; RNP, ribonucleoprotein; Sm, Smith; TRIM21, TRIPartite Motif protein (TRIM) 21.
¹dsDNA was measured at enrolment for only 26 patients on ELISA, 13 on IFA2, and 2 on both who tested within the normal range for ANA

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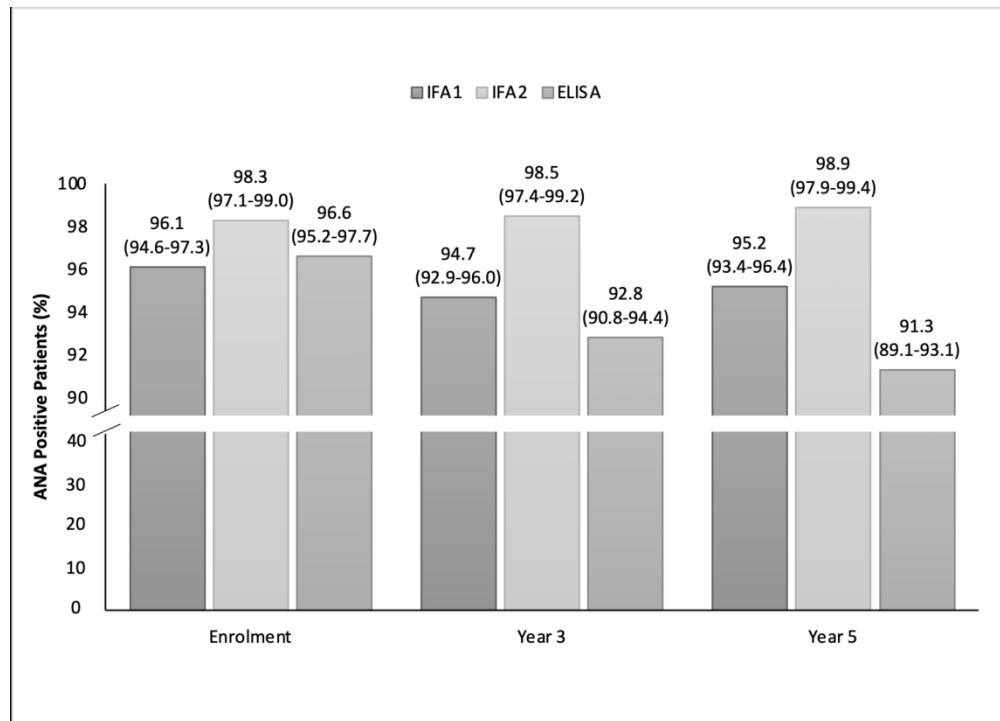


Figure 1. ANA positivity among IFA1 (n=805), IFA2 (n=805) and ELISA (n=805) at enrolment, year 3 and year 5. There is a break in the y-axis between 40% and 90% to enhance the readability of the graph from 90-100%.

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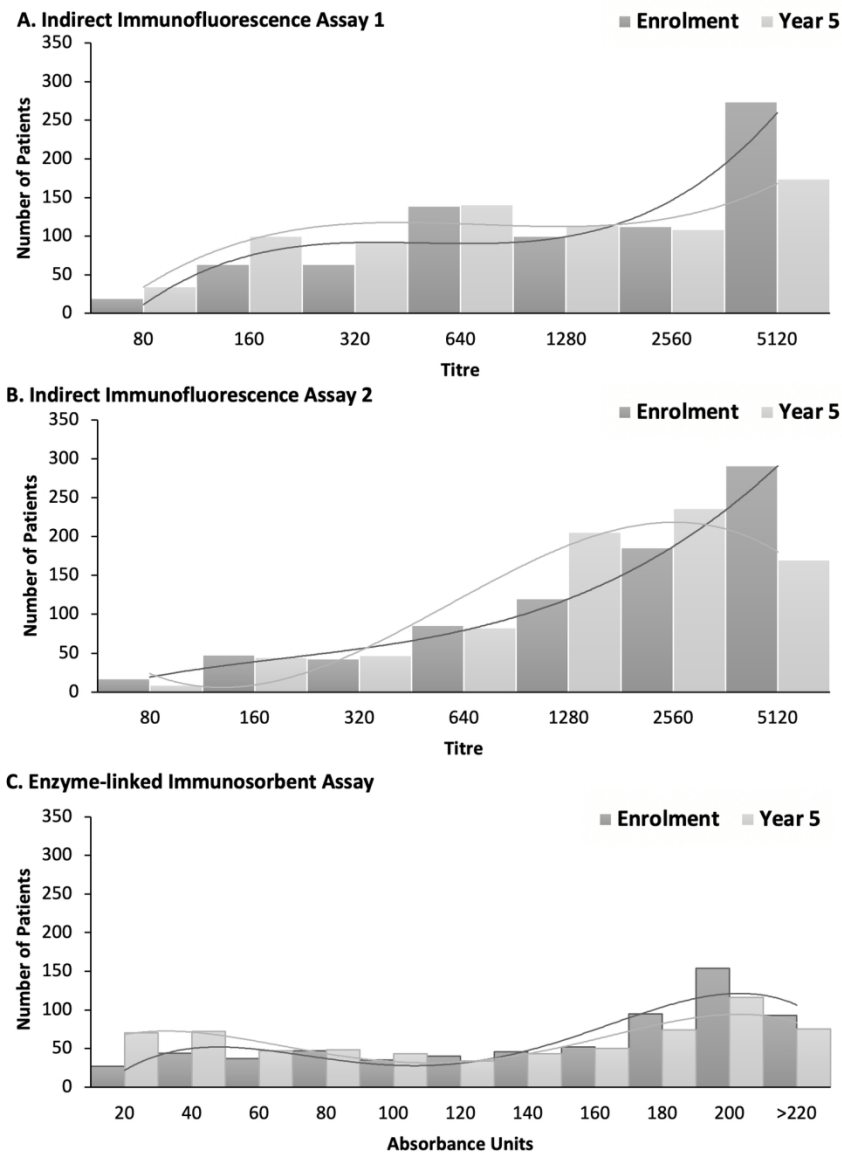


Figure 2. Distribution of ANA titres at enrolment and year 5 visit for A) indirect immunofluorescence 1 (IFA1) (n=805), B) IFA2 (n=805) and C) enzyme-linked immunosorbent assay (ELISA) (n=805). Lines represent the curve of best fit.

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Supplemental Table 1. SLE-related Autoantibodies performed

Autoantibody	Assay and Cut-offs
Anti-dsDNA	Anti-dsDNA positivity and titers were detected by a chemiluminescence immunoassay (CLIA) (Werfen, San Diego, USA). A cut-off of ≥ 27 chemiluminescence units (CU) was utilized, where 27-35 (CU) was indeterminate (borderline), and >35 was positive.
Other SLE-specific autoantibodies	Performed using ALBIA (FIDIS Connective13; TheraDiag, Paris, France) on a Luminex 200 flow luminometer (Luminex, Austin, USA) focussing on SLE-related analytes that included ribosomal P, Ro52/Tripartite Motif Protein 21 (TRIM21), SSA/Ro60, SSB/La, Sm, U1-RNP, and histones. A cut-off of >40 median fluorescence units (MFU) was considered positive.
Anti-phospholipid antibodies	Anti-phospholipid antibodies including IgG and IgM anticardiolipin and anti- $\beta 2$ -glycoprotein-1 were measured using ELISA (Werfen, San Diego, USA). Using the revised Sapporo antiphospholipid syndrome classification criteria (1), a cut-off of >40 units for IgG/IgM anti-cardiolipin was considered medium to high positive while a cut-off of ≥ 20 units ($>99^{\text{th}}$ percentile) was positive for IgG/IgM anti- $\beta 2$ -glycoprotein-1 ($\beta 2$ GP1) (1). All autoantibodies were measured at MitogenDx except for lupus anticoagulant, which was measured at Oklahoma Medical Foundation (Oklahoma City, OK) as previously described (2).

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12 **Supplemental Table 2. Clinically defined samples**

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Demographic and Clinical Variables	Definition
Age	Years at diagnosis
Sex	Female or male
Race/ethnicity	Asian: Chinese, Filipino, Japanese, Korean, other Asians; African descendants: African, Caribbean; Hispanic: Hispanics only; White: North American, Indian – sub-continent, other Caucasians; Other races/ethnicities: Native North American, Native Hawaiian or other Pacific Islanders, others
Presence of nephritis	Based on renal biopsy or fulfillment of the renal item of the ACR Classification criteria
American College of Rheumatology (ACR) criteria	Number of specific ACR criteria fulfilled
SLE Disease Activity Index (SLEDAI-2K) score	Disease activity measured by global SLEDAI-2K score and its individual components grouped to represent the following organ systems: Neurological: seizures, psychosis, organic brain syndrome, visual disturbance, cranial nerve disorder, lupus headache, CVA Mucocutaneous: vasculitis, rash, alopecia, mucosal ulcers Musculoskeletal: arthritis, myositis Renal: urinary casts, hematuria, proteinuria, pyuria Serositis: pleurisy, pericarditis Constitutional: fever Immunological: low complement, increased DNA binding Hematological: thrombocytopenia, leukopenia
Medications	Any use of oral or parental glucocorticoids, antimalarials, immunosuppressive agents (methotrexate, azathioprine, mycophenolate, cyclophosphamide, cyclosporine, and biologics) at or prior to enrollment.

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15 **Supplemental Table 3. Patient characteristics at enrolment comparing patients included in**
 16 **this study and the remaining SLICC pts providing at least 4 yrs of clinical data, but for**
 17 **whom 3 serial samples were not available**
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Characteristic	Study Cohort n=805	Remainder of SLICC n=627	Difference (95% CI) ¹
Mean age at dx, yrs (SD)	35.2 (13.6)	34.1 (13.0)	1.1 (-0.3, 2.4)
Female, %	88.7	88.5	0.2 (-3.1, 3.5)
Mean disease duration, yrs (SD)	0.58 (0.49)	0.49 (0.35)	0.1 (0.05, 0.14)
Mean (SD) number of ACR Criteria excluding the ANA	3.9 (1)	4 (1.1)	-0.1 (-0.2, 0)
Ethnicity, %			
Asian	24.3	5.6	18.8 (15.3, 22.2)
African	13.5	18.3	-4.8 (-8.6, -1)
White	52.3	45.1	7.2 (2, 12.4)
Hispanics	6.3	27	-20.6 (-24.5, -16.8)
Other ethnicity ²	3.5	4	-0.5 (-2.5, 1.5)
Nephritis ³	28.9	33.3	-4.4 (-9.2, 0.5)
Mean total SLEDAI-2K (SD)	5.4 (5.3)	5.2 (5.46)	0.2 (-0.4, 0.8)
Mean total SDI (SD)	0.34 (0.74)	0.35 (0.8)	-0.01 (-0.13, 0.11)
Current medications, %			
Glucocorticoids	69.6	71	-1.4 (-6.2, 3.4)
Antimalarials	70.1	67.3	2.8 (-2.1, 7.6)
Immunosuppressants	41	43.1	-2.1 (-7.2, 3.1)
Medications ever, %			
Glucocorticoids	81.5	82.1	-0.6 (-4.7, 3.4)
Antimalarials	76.6	73.5	3.1 (-1.4, 7.6)
Immunosuppressants	43.9	46.3	-2.4 (-7.6, 2.8)
Autoantibodies, %			
Lupus anticoagulant	19.7	19.8	-0.1 (-5.6, 5.3)

Abbreviations: ACR, American College of Rheumatology; ANA, anti-nuclear antibodies; CI, confidence interval; x, diagnosis; SD, standard deviation; SLEDAI-2K, systemic lupus erythematosus disease activity index-2000; SDI, SLICC Disease index; yrs, years.

1. Difference between study cohort and remainder of SLICC cohort

2. Other ethnicities include: Native North American, Native Hawaiian or other Pacific Islanders

3. Nephritis defined as fulfilling the ACR criterion for renal disease or if a renal biopsy was performed prior to cohort entry

25 **Supplemental Table 4. Patient characteristics at enrolment comparing patients providing**
 26 **3rd sample between 4 and 7 yrs of follow-up and pts providing 3rd sample between 8 and**
 27 **10 yrs of follow-up. Bolded indicates statistically significant p<0.05.**
 28

Characteristic	Patients without delayed year 5 visit N=781	Patients with delayed year 5 visit N=24	Difference (95% CI) ¹
Demographic and Clinical			
Mean age at dx, yrs (SD)	35.2 (13.6)	32.8 (14.1)	2.4 (-3.3, 8.1)
Female, %	88.7	87.5	1.2 (-12.2, 14.6)
Mean disease duration, yrs (SD)	0.58 (0.49)	0.76 (0.63)	-0.18 (-0.44, 0.07)
Meeting ACR Criteria without ANA, %	3.9 (1)	3.9 (1.02)	0 (-0.4, 0.4)
Ethnicity, %			
Asian	24.6	16.7	7.9 (-7.3, 23.1)
African	13.6	12.5	1.1 (-12.4, 14.5)
Caucasian	52.4	50	2.4 (-17.9, 22.7)
Hispanic	6.1	12.5	-6.4 (-19.7, 7)
Other ethnicities ²	3.3	8.3	-5 (-16.1, 6.1)
Nephritis ³	28.9	29.2	-0.2 (-18.7, 18.2)
Mean total SLEDAI-2K (SD)	5.4 (5.3)	4.3 (4.03)	1.1 (-0.6, 2.7)
Mean total SDI (SD) ⁴	0.33 (0.73)	0.67 (0.89)	-0.34 (-0.85, 0.17)
Medications			
Current, %			
Glucocorticoids	69.4	75	-5.6 (-23.2, 12)
Antimalarials	70.2	66.7	3.5 (-15.6, 22.6)
Immunosuppressants	41	41.7	-0.7 (-20.7, 19.3)
Ever, %			
Glucocorticoids	81	95.8	-14.8 (-23.2, -6.3)
Antimalarials	77	66.7	10.3 (-8.8, 29.4)
Immunosuppressants	43.8	45.8	-2 (-22.3, 18.2)
Autoantibodies, %			
DsDNA ⁵	34.4	25	9.4 (-8.3, 27)
Ribosomal P	24.7	12.5	12.2 (-1.4, 25.8)
Ro52/TRIM21	37.3	45.8	-8.6 (-28.8, 11.6)
SSA/Ro60	42.6	37.5	5.1 (-14.5, 24.8)
SSB/La	21	12.5	8.5 (-5, 22)
Sm	23.2	8.3	14.8 (3.4, 26.3)
U1RNP	28.4	20.8	7.6 (-9, 24.1)
Histones	31.8	16.7	15.1 (-0.2, 30.4)
Cardiolipin IgG/IgM ⁶	20.6	16.7	4 (-11.2, 19.1)
β2GP1 IgG/IgM ⁶	20.1	8.3	11.8 (0.4, 23.2)

Lupus anticoagulant ⁷	20.1	5.3	14.8 (4.3, 25.3)
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Abbreviations: ACR, American College of Rheumatology; ANA, anti-nuclear antibodies; β 2GP1, β 2-glycoprotein-1; CI, confidence interval; dx, diagnosis; dsDNA, double-stranded DNA; IgG/M, immunoglobulin G/immunoglobulin M; RNP, ribonucleoprotein; SD, standard deviation; SLEDAI-2K, systemic lupus erythematosus disease activity index-2000; SDI, SLICC Disease index; TRIM21, Tripartite Motif Protein (TRIM) 21; yrs, years.

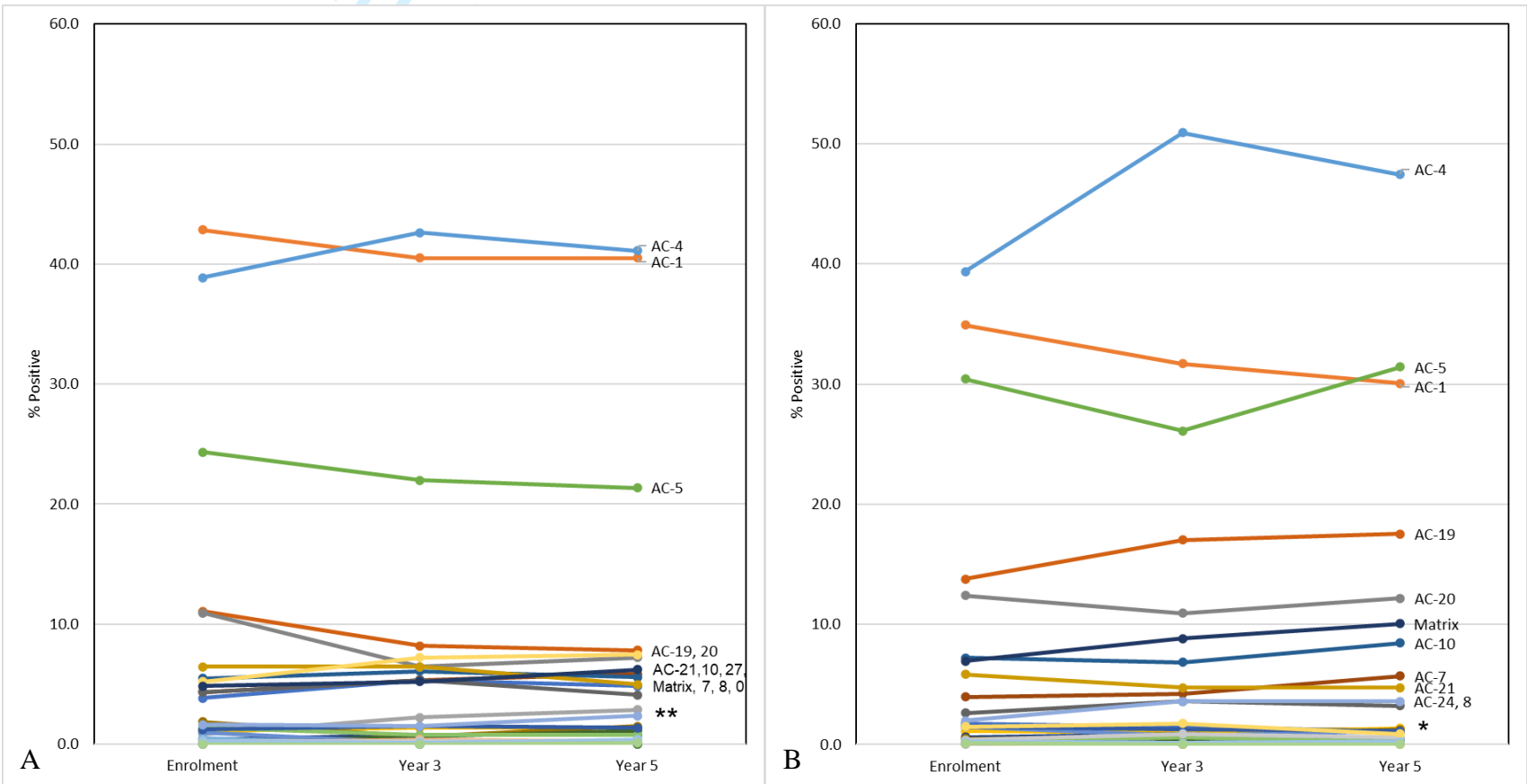
1. Difference between enrolment and year 5 visit
2. Other ethnicities include: Native North American, Native Hawaiian or other Pacific Islanders
3. Nephritis defined as fulfilling the ACR criterion for renal disease or if a renal biopsy was performed prior to cohort entry
4. Data available for n= 368, 781, 780 at enrolment, year 3, and year 5 respectively
5. Data available for n= 777, 778, 778 at enrolment, year 3, and year 5 respectively
6. Data available for n= 776, 781, and 781, at enrolment, year 3, and year 5 respectively
7. Data available for n= 647, 469, and 288, at enrolment, year 3, and year 5 respectively

42 **Supplemental Table 5. Pairwise conflicting results between IFA1, IFA2, and ELISA (total n at each time point = 805)**

	+IFA1 -IFA2 (n)	-IFA1 +IFA2 (n)	p-value	+IFA1 -ELISA (n)	-IFA1 +ELISA (n)	p-value	+IFA2 -ELISA (n)	-IFA2 +ELISA (n)	p-value
Enrolment	6	23	<0.05	19	23	NS	24	11	<0.05
Year 3	4	35	<0.001	43	28	NS	53	7	<0.001
Year 5	3	33	<0.001	50	19	<0.001	66	5	<0.001

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45 Abbreviations: ANA, anti-nuclear antibodies; ELISA, enzyme-linked immunosorbent assay; IFA; indirect immunofluorescence assay;
46 NS, non-significant. **p<0.001, *p<0.05 for significant asymmetry using McNemar's Test

48 **Supplemental Figure 1. Frequency of IFA1 (A) and IFA2 (B) ANA on HEp-2 ICAP patterns AC0-AC29, matrix at enrolment,**
 49 **year 3, and year 5. ** Represents AC- 2-3, 6, 9, 11-18, 22-26, 28-29, * represent AC-0, 2-3, 6, 9, 11-18, 22-23, 25-29. Refer to the**
 50 **ICAP website (www.anapatterns.org) for detailed descriptors for each AC pattern. For IFA1 or IFA2, the most common**
 51 **patterns: AC-4 represents nuclear fine speckled, AC-5 nuclear large speckled, AC-1 nuclear homogeneous, AC-19 cytoplasmic**
 52 **dense fine speckled, AC-20 cytoplasmic fine speckled, AC-matrix is matrix, AC-10 punctate nucleolar, AC-7 few discrete**
 53 **nuclear dots, AC-21 cytoplasmic reticular/anti-mitochondrial antibodies, AC-8 homogeneous nucleolar, AC-24 centrosome,**
 54 **AC-27 intracellular bridge, AC-0 negative/no pattern observed.**
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